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CHANGES IN THE ENZYMATIC CONSTITUTION OF THE VENTRAL PROSTATE GLAND INDUCED BY ANDROGENIC HORMONES

H. G. WILLIAMS-ASHMAN[1]

The Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Illinois

SOME enzymatic activities in intact and broken cell preparations of canine and human prostate tissue have been investigated by Barron and Huggins (1944, 1946, a, b). The striking effects of androgens on the respiration of canine prostate (Barron and Huggins, 1944) have also been demonstrated in the ventral prostate gland of the rat (Nyden and Williams-Ashman, 1953). The enzymatic constitution of the latter organ is relatively unexplored. Davis, Meyer and McShan (1949) have measured the succinic oxidase and cytochrome oxidase activities of this tissue and Awapara (1952 a) has reported measurements of certain transaminase activities. Rudolph and Starnes (1952) have studied the oxidation of acetate-1-C^{14} by rat prostate slices.

This paper will describe measurements of the activity of a number of enzymes in the rat ventral prostate and changes in these values induced by androgens. In view of the postulated absence of the tricarboxylic acid cycle in prostatic tissue (Rudolph and Starnes, 1952; Awapara, 1952 b) and of the intense accumulation of citric acid which results from androgenic stimulation (Mann and Parsons, 1950), attention has been given toward the enzymes of the tricarboxylic acid cycle and especially those involved in the degradation of citric acid viz. aconitase and isocitric dehydrogenase. The enolase activity of this organ has also been investigated in the light of the

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[1] Scholar in Cancer Research of the American Cancer Society.

alleged influence of androgenic hormones on this enzyme (Kun, 1950; Dirscherl and Hauptman, 1950).

METHODS

Commercial samples of DPN[2] (purity of 0.53 to 0.58) were purified chromatographically on Dowex-1 resins according to an unpublished procedure of A. Kornberg and B. L. Horecker. The purity of the samples used ranged from 0.80 to 0.87. TPN (purity 0.62) was obtained from the Sigma Chemical Co. DPNH was prepared essentially according to Ohlmeyer (1938). 2-P-G was synthesized by the method of Kiessling (1935). All other materials were obtained commercially.

Acetone powders were prepared as follows: The ventral prostate tissue was removed immediately after killing the animals by cervical fracture, and dissected free of as much fat and connective tissue as possible. The glands were placed in iced water and cooled in an ice bath for 30 minutes. The tissue was homogenized in an all glass apparatus immersed in an ice-salt bath, approximately 20 per cent homogenates being prepared. Five volumes of acetone (−25°) were added with stirring, and the mixture centrifuged at −5°. The precipitate was washed three times with acetone and dried *in vacuo* over $CaCl_2$. In preparing the extracts the dry powder was pulverized, weighed and extracted by trituration with 30 volumes of 0.01 M $NaHCO_3$ at 0° for one hour. The mixture was centrifuged for 30 minutes at 5,000 × g at 0°, the resulting clear supernatant fluid being used for the determinations of enzyme activity.

The animals, diets, tube feeding regime and surgical techniques employed are described elsewhere (Nyden and Williams-Ashman, 1953).

Spectrophotometric determinations of enzyme activities were performed with a Beckman D.U. spectrophotometer using quartz cells of 1 cm. light path. All estimations were performed at 27–29°. Unless otherwise stated the reactions were initiated by the addition of enzyme. Blank cells containing all the reactants except the substrate were always run in order to compensate for any changes in optical density due to endogenous substrates etc. All values recorded are corrected for such changes. No turbidity was present in any of the assays described. The volume and composition of the test solutions are described under the relevant protocols.

Protein was determined spectrophotometrically according to Warburg and Christian (1943) and nitrogen by the Kjeldahl technique. DPN was estimated with the aid of alcohol dehydrogenase and ethanol at pH 9 (Racker, 1950 a) and TPN was determined with purified G-6-P dehydrogenase (Kornberg, 1950).

RESULTS AND DISCUSSION

Determination of enzyme activities in acetone powder extracts

Initial attempts to measure the activity of a number of prostatic enzymes by spectrophotometric tests using unfractionated and centrifuged homogenates of fresh tissue were largely unsuccessful. The turbidity of such preparations proved to be very troublesome. Estimations of the reduc-

[2] The following abbreviations are employed: DPN = diphosphopyridine nucleotide; TPN = triphospho-pyridine nucleotide; DPNH = dihydrodiphosphopyridine nucleotide; G-6-P = glucose-6-phosphate; 2-P-G = 2-phosphoglycerate; TPNH = dihydrotriphosphopyridine nucleotide.

tion of pyridine nucleotides, or the oxidation of reduced pyridine nucleotides, by various substrates were severely complicated by the presence of enzymes which catalyzed the destruction of pyridine nucleotides, by powerful DPNH and TPNH oxidases, and by the reduction of pyridine nucleotides by endogenous substrates.

Acetone powder extracts were found to be suitable for studies of most of the enzymes described in this paper. Under the extraction conditions employed the amounts of protein and nitrogen extracted from one powder to another were remarkably similar with ventral prostate tissue derived from

TABLE 1. EXTRACTION OF PROTEIN FROM PROSTATE ACETONE POWDERS

Treatment of animals	$\frac{E_{280}}{E_{260}}$*	Mg. protein per cc.	Mg. nitrogen per cc.
Sham operated	1.10	11.2	1.86
Castrated	1.14	11.9	1.93
Castrated and treated with testosterone propionate	0.96	11.5	1.96

* Ratio of optical density at 280 mμ to that at 260 mμ in 0.033 M sodium phosphate buffer pH 7.4.

Powders obtained from Series 1 of Table 2.

normal and castrated animals, and castrated animals treated with androgens (Table 1). The enzyme activities observed are given in terms of units per mg. protein, but the relative values obtained were virtually the same if expressed in terms of either units per mg. nitrogen or units per mg. dry weight of acetone powder. In each series of experiments tissue was pooled from a number of animals which tended to iron out differences between the activity of the individual members of each group. In all measurements of enzyme activity the final dilution of the extracts were such that the rates measured were directly proportional to the amount of protein added and the reactions were of zero order over the periods used to calculate the rates. A typical plot of rate of reaction with different enzyme concentrations for ventral prostate aconitase, with both citrate and isocitrate as substrates, is shown in Figure 1. In all the optical tests the concentration of substrates and cofactors were optimal; increasing them two fold did not significantly alter the rates observed.

Influence of androgens upon glycolytic and respiratory enzymes in rat ventral prostate

Values for the activity of a number of glycolytic and respiratory enzymes in ventral prostate tissue of normal rats, and of castrated animals with or without further treatment with testosterone propionate are shown in Tables 2, 4 and 6. The activity of the pyridine nucleotide linked dehydro-

TABLE 2. INFLUENCE OF ANDROGENS ON PROSTATIC HYDRASES

Series	Units per mg. protein*		
	S	C	C+T
Fumarase			
1	36.2	28.2	40.2
2	51.5	30.4	50.1
3	31.5	24.3	39.5
Mean	39.7	27.6	43.3
Aconitase			
1	24.0	12.4	26.1
2	26.7	14.1	25.4
Mean	25.3	13.5	25.7
Enolase			
1	30.8	40.2	33.2
2	37.0	37.1	41.9
Mean	33.9	38.7	37.5

* Reaction carried out in final volume of 3.0 cc. at 27°, and initiated by the addition of extract. Each cell contained 1.0 cc. 0.1 M sodium phosphate pH 7.4 and the following constituents for measurement of various enzyme activities: Fumarase; 100 μM sodium DL-malate; Aconitase: 90 μM sodium citrate; Enolase: 30 μM $MgCl_2$ and 25 μM sodium 2-phosphoglycerate. One unit = change in optical density of 0.001 per minute, wavelength 240 mμ. S = Sham operated; C = castrated; C+T = Castrated treated with 1 mg./day testosterone propionate. Acetone powders prepared seven days after surgery. Four animals in each group in each series of experiments.

genases was followed by change in optical density at 340 mμ, while changes in light absorption at 240 mμ were employed to determine enolase activity according to Warburg and Christian (1943) and fumarase and aconitase activities according to Racker (1950 b). Although the experimental conditions were somewhat different, the activities of fumarase, aconitase, malic dehydrogenase and isocitric dehydrogenase fell well within the range shown for a number of other rat tissues observed by Wenner *et al.* (1952). Remarkably low values for the activity of lactic dehydrogenase were observed compared with those reported for most tissues (Wenner *et al.*, 1952; Meister, 1950). Values for the activity of G-6-P dehydrogenase are shown in Table 5.

TABLE 3. STABILITY OF PROSTATIC HYDRASES

	Enzyme activity*		
	Fumarse	Aconitase	Enolase
Sham operated	85	37	97
Castrated	90	41	99
Castrated treated with testosterone propionate†	82	30	80

* Per cent of that exhibited by fresh extract after storage for 20 hours at 2°.
† 1 mg. per day.

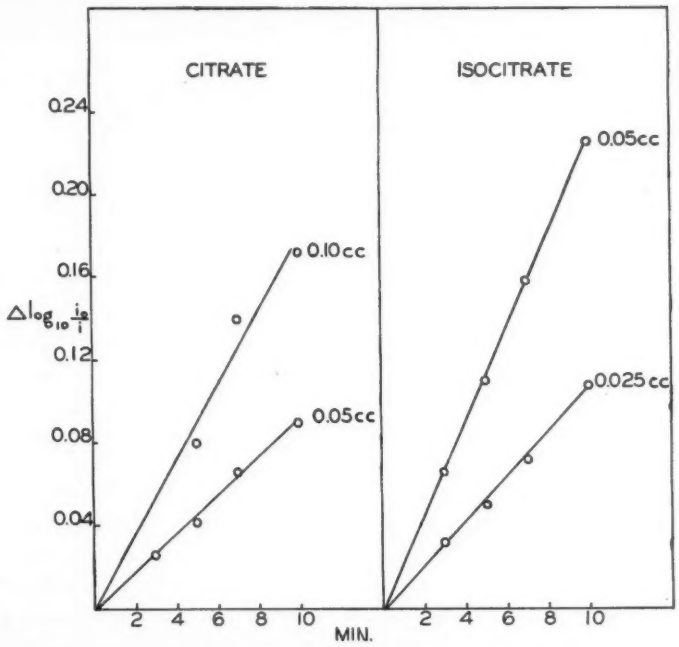

FIG. 1. Spectrophotometric determination of ventral prostate aconitase. Each cell contained 150 μM sodium phosphate pH 7.4 and final volume of 3.05 cc. Substrate either 30 μM sodium citrate or 30 μM sodium DL-isocitrate. Reaction initiated by the addition of extract, volumes depicted. The extract contained 12.1 mg. protein per cc. Temp. 22°. Wavelength 240 mμ.

Marked activation of prostatic isocitric dehydrogenase was observed on the addition of Mn^{++} ions in a manner similar to that described by Ochoa (1948) for this enzyme in pig heart. Figure 2 depicts the activation of iso-

TABLE 4. ISOCITRIC DEHYDROGENASE

Series	Treatment of animals	Units per mg. protein	
		Mn^{++}	No Mn^{++}
3	Sham operated	243	89
3	Castrate	226	39
3	Castrate treated with testosterone propionate	456	119
4	Unoperated	285	78
4	Castrate	234	40

Reactions carried out in a final volume of 1.60 cc., containing 50 μM glycyl-glycine pH 7.4; 0.2 μM TPN and 10 μM sodium DL-isocitrate. $MnCl_2 = 1$ μM if added. Reaction initiated by the addition of 0.02 cc. of 1:30 acetone powder extract. 1 unit = change in optical density of 0.001 per minute at 27°, corrected for any optical density change in the absence of substrate. Wavelength 340 mμ. Acetone powder prepared from group of four animals seven days after surgery. Testosterone propionate 1 mg./day.

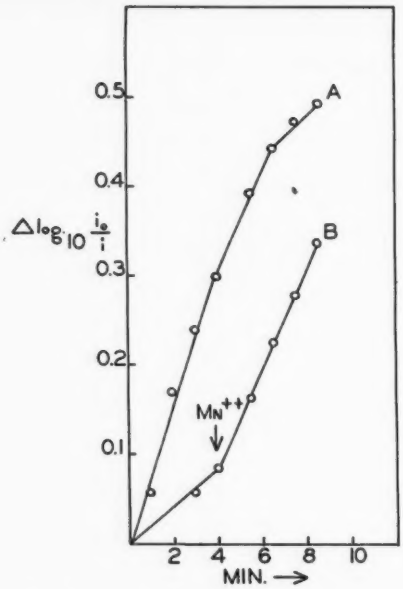

FIG. 2. Activation of ventral prostate isocitric dehydrogenase by Mn^{++} ions. Each cuvette contained 50 μM glycyl-glycine pH 7.4; 0.2 μM TPN; 10 μM sodium DL-isocitrate and 0.02 cc. extract (0.26 mg. protein) in a final volume of 1.60 cc. Curve A: 0.05 cc. $MnCl_2$ (1 μM) added before extract. Curve B: 0.05 cc. $MnCl_2$ (1 μM) added at arrow. Wavelength 340 mμ. Temperature 27°.

citric dehydrogenase of normal rat ventral prostate by Mn^{++} ions added either at the start of the experiment or at later time intervals. A similar activation of the enzyme was observed with preparations from castrated and castrated-androgen treated animals (Table 4). These experiments argue for the presence of oxalosuccinic carboxylase in ventral prostate (cf. Ochoa, 1948).

Seven days after castration, a marked decrease in the activity of fumarase and aconitase activity occurred, whereas enolase activity was, if anything, enhanced by this treatment (Table 2). Administration of testosterone propionate to castrated animals reversed these changes. An examination of the stability of these hydrating enzymes to storage at 2° showed that, with all types of preparation, the fumarase and enolase activity of the undiluted extracts was relatively stable. The marked decrease in aconitase activity which occurred on storage was of the same order of magnitude with extracts from normal, castrated and castrated-androgen treated animals (Table 3).

TABLE 5. GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Series	Treatment of animals	Units/mg. protein
3	Sham operated	15.0
3	Castrated	15.3
3	Castrated and treated with testosterone propionate	14.6
4	Unoperated	12.9
4	Castrated	16.7

Conditions as in Table 4 except that substrate consisted of 10 μM G-6-P. 1 μM $MnCl_2$ in all vessels. Reactions initiated by addition of 0.10 cc. of 1:30 acetone powder extract.

Tables 4 and 5 show that castration had little influence on isocitric dehydrogenase or glucose-6-phosphate dehydrogenase. However, this treatment induced an unmistakable increase in lactic dehydrogenase activity and decrease in malic dehydrogenase (Table 6).

In all experiments involving castration the animals were tube-fed, so that alterations in the dietary intake could not be responsible for the effects observed.

Many attempts were made to influence the activity of these enzymes by the *in vitro* addition of androgenic steroids. Since the results of these experiments were negative, actual experimental details will not be given. At a final concentration of 10^{-6} M and 10^{-5} M testosterone failed to affect the activity of fumarase, aconitase, enolase, lactic dehydrogenase, isocitric dehydrogenase and glucose-6-phosphate dehydrogenase. Numerous experiments with rat ventral prostatic enolase, measured in the absence of Mg^{++} ions, or in the presence of suboptimal and optimal Mg^{++} ion concentrations failed to show any significant effect of testosterone on this enzyme, as has been claimed by Dirscherl and Hauptman (1950). Similarly crude and par-

TABLE 6. LACTIC AND MALIC DEHYDROGENASES

Series	Units per mg. protein*		
	S	C	C+T
Lactic dehydrogenase			
1	35.0	51.6	40.2
2	30.4	35.2	22.6
Mean	32.7	43.4	31.4
Malic dehydrogenase			
1	290	198	298
2	183	113	187
Mean	236	155	242

Reactions carried out in a final volume of 3.0 cc. at 27–28° and initiated by the addition of extract. Each cell contained 1.0 cc. of 0.1 M sodium phosphate pH 7.4 and 0.38 μM DPNH. In the lactic dehydrogenase test the substrate was pyruvate (15 μM) and in the malic dehydrogenase test the substrate was oxalacetate (20 μM). Animals treated as in Table 2.

* 1 unit = change in optical density of 0.001 per minute at 340 mμ.

tially purified preparations of enolase from human prostatic adenoma were uninfluenced by this hormone.

The enzymatic activities recorded in this paper suggest the operation of the tricarboxylic acid cycle in ventral prostate tissue, and the inhibitory influence of 2,4 dinitrophenol on a number of synthetic reactions in respiring ventral prostate slices (Nyden and Williams-Ashman, 1953) indicates the occurrence of attendant oxidative phosphorylations. This conclusion is corroborated by experiments of Williams-Ashman (1953) who has shown that ventral prostate mitochondria will catalyze the following reactions provided they are supplemented with pyridine nucleotides and coenzyme A: (i) synthesis of citrate from pyruvate and oxaloacetate, (ii) oxidation of added citrate, (iii) oxidation of α-ketoglutarate. Furthermore the presence of condensing enzyme and of the ATP-acetate reaction could be directly demonstrated in extracts of ventral prostate acetone powders.

SUMMARY

A number of glycolytic and respiratory enzymes have been assayed in the ventral prostate gland of the rat. The results obtained strongly indicate the presence of the tricarboxylic acid cycle in this tissue.

Castration leads to a decrease in the activity of aconitase, fumarase and malic dehydrogenase and an enhanced lactic dehydrogenase activity, which can be reversed to normal levels by the administration of testosterone propionate. Isocitric dehydrogenase, glucose-6-phosphate dehydrogenase and enolase activity are not significantly affected.

None of the enzyme systems were affected by the *in vitro* addition of testosterone.

Acknowledgment

The author is indebted to Dr. S. J. Nyden and Mrs. J. Banks for assistance in some of these experiments.

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COMPARISON OF THE INFLUENCES OF ADRENOCORTICAL HORMONES ON THE GROWTH AND INVOLUTION OF LYMPHATIC ORGANS[1,2,3]

GEORGE A. SANTISTEBAN[4] AND THOMAS F. DOUGHERTY

Department of Anatomy and Radiobiology Laboratory, University of Utah College of Medicine, Salt Lake City, Utah

PRELIMINARY investigations of the acute involution of lymphatic organs induced by administration of cortisone demonstrated that the hormone-induced involution of lymphatic tissue was a response of the continuous variate type (Santisteban, 1953). Accordingly, more detailed studies were performed to ascertain whether the regression relationships of certain lymphatic organs could be employed to elucidate further the quantitative interrelationships of amounts and molecular structures of certain adrenocortical hormones to the growth and involution of lymphatic organs. Although the method presented here might be adapted to various types of bioassay procedures, the emphasis in these studies has been directed towards establishing the comparative potencies and structural activity relationships of those adrenocortical hormones which were known to have the capacity to induce acute lymphatic organ involution (reviewed by Dougherty, 1952). Other steroid hormones which have been reported by various authors to produce growth of lymphatic organs were also included in the present study.

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[2] The authors wish to thank Dr. Augustus Gibson of Merck and Company for the generous supply of cortisone and hydrocortisone and Dr. Abraham White formerly of Chemical Specialties for the other compounds used in these investigations. We also wish to thank Dr. Lowell Woodbury for his aid and advice in planning the statistical methodology employed in these studies.

[3] This paper is a part of the content of a dissertation submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Anatomy of the Graduate School of the University of Utah, August, 1951.

[4] Present address: Department of Anatomy, Medical College of Virginia, Richmond, Virginia.

MATERIALS AND METHODS

A total of 56 female CBA mice, 10 weeks of age, (18.2 ±0.5 gm. body weight), were used for the assay of each of the various compounds. They were divided into 7 groups, each of which contained 8 animals. Two groups served as non-treated controls, one intact and one adrenalectomized. All the animals in the remaining five groups were adrenalectomized and treated with graded doses of the compound to be assayed.

The following steroid hormones were used in the experiments: Compound F Acetate (hydrocortisone acetate; 17-hydroxycorticosterone acetate, Compound E Acetate (cortisone acetate; 17-hydroxy-11-dehydrocorticosterone acetate), Compound A Acetate (11-dehydrocorticosterone acetate, Compound B Acetate (corticosterone acetate), Substance L Acetate (allo-Pregnane-3(β):17(β):diol-20-one acetate), Substance S (11-desoxy-17-hydroxycorticosterone) and desoxycorticosterone glucoside. With the exception of desoxycorticosterone glucoside the compounds were all suspended in the suspending agents used for commercial preparations of cortisone (Cortone, Merck). The suspended hormones were diluted with appropriate amounts of sterile non-pyrogenic saline made up into constant volumes of 0.2 cc. The doses used were 5, 25, 50, 250 and 500 μg./day. These were given by intraperitoneal injections, which were started 20–24 hours after the adrenalectomy, and subsequently were given in divided doses twice daily (12-hour intervals), for 4 successive days.

The animals were sacrificed on the fifth day, 12 hours after the last injection. Initial and final body weights were recorded for all experimental and control animals. The wet weights of paired peripheral lymph nodes (axillary and inguinal), mesenteric lymph nodes, thymi and spleens were determined to the nearest 0.5 mg. on a Roller-Smith torsion balance. The mean relative weights (mg./100 gm. initial body weight) of each of these organs were plotted against the log-dose of the hormone. Sigmoid log-dose response curves were obtained for the alterations in weight of all lymphatic organs. The rectilinear portions of the curves fell within the dose range of 25 to 500 micrograms and these segments of the curves for the thymi and peripheral lymph nodes were mathematically fitted according to the method of least squares. The details of the mathematical procedures used here have been presented by Irwin (1937), Pugsley (1946), Emmens (1948) and Sayers *et al.* (1948).

Histological investigations were performed on the same organs which were weighed. Comparison of the cytological effects of hydrocortisone and cortisone were made on air-dried imprints stained with May-Grünwald Giemsa and on Zenker formol fixed tissues stained with methyl green pyronin.

RESULTS

Thymus

Table 1 presents the results of the assays of the various steroid hormones based on their effects on the weight of the thymus. During a period of 9 months, three separate tests were performed using cortisone (E_1, E_2, E_3) at intervals of 3 months. Two experiments were completed using Compounds B (B_1, B_2) and S (S_1, S_2). Each tabulated value represents the mean weight of the thymus expressed in mg./100 gm. initial body weight. In order to establish the mean and standard error of the total intact and adrenalectomized control groups, the means for these controls from each

TABLE 1. MEANS OF RELATIVE WEIGHTS* OF THYMI OF ADRENALECTOMIZED MICE TREATED WITH GRADED DOSES OF VARIOUS STEROID HORMONES

Compound	Control non-treated		Adrenalectomized-hormone treated dose in micrograms/day†						
	Intact	Adrenalectomized	5	25	50	250	500	750	1000
E_1	256.0	290.5	305.0	265.2	170.0	138.0	78.2		
E_2	186.0	278.0	270.8	243.9	255.7	104.7	83.6		
E_3	315.1	267.3	266.0	274.2	244.3	155.8	97.2		
B_1	246.8	279.8	197.0	254.4	304.3	80.8	140.2		
B_2	252.5	250.2	258.2	280.2	269.9	155.9	101.6		
S_1	225.1	312.3	304.6	271.7	311.2	288.7	279.7		
S_2	249.1	306.9	—	—	305.3	324.9	* 295.3	296.8	285.0
F	264.5	265.0	226.2	217.4	170.0	38.9	34.3		
L	271.3	281.4	281.3	260.2	276.5	264.6	265.5		
A	275.9	268.6	287.1	286.1	257.1	183.3	141.0		
DCG	192.3	342.3	268.4	305.1	332.1	340.6	329.8		
Means of control groups	248.6	285.7							
S.E.	±11.7	±8.2							
P‡	<0.02								

$$\text{S.E.} = \frac{\text{S.D.}}{\sqrt{N}} = \text{Standard error of means}$$

* Mg./100 gm. initial body weight.
† Treated for 4 days after adrenalectomy.
‡ P value for the difference between the means of the intact and adrenalectomized controls.

experiment were treated as single observations. The significant hypertrophy of the thymus which was found 4 days after adrenalectomy is indicated by the difference of the means between the two control groups ($P > .02$). Involutionary effects were obtained with Compounds E, B, A and F; a possible growing effect was obtained with DCG; but Compounds L and S had no effect.

The slopes of the log-dose response lines for each assay as fitted mathematically from the data presented in Table 1 are given in Table 2. These lines were fitted according to the equation $y = a + bx$, where y = the response expressed as the weight of the thymus in mg./100 gm. initial body weight; x = log of the dose; a = the y intercept; and b = the slope of the line. Negative slopes indicate involution and positive slopes indicate growth.

The standard deviations about the lines S.D.L, the standard errors of the slopes S.E.$_b$ and values of λ (indices of precision) are also presented for each fitted line and for the combined lines E_c ($E_1+E_2+E_3$) and B_c (B_1+B_2). The table also includes these same statistical quantities (S.D.L$_c$, S.E.b_c, λ_c) as computed for the lines E_c, A, F and B_c fitted to a common slope b_c. The values of λ for each separate line indicate that this method for evaluating the activity of graded doses of purified crystalline steroid compounds has a relatively high degree of accuracy. Despite the high values of λ for some of the assays (E_1, B_1, A) the value λ for b_c indicates an adequate level of accuracy for the method. The values λ_c (for each of the lines with slopes b_c) indicate a high degree of precision (with the exception of B_c) even after fitting the lines to common slope for the estimation of potency ratios.

The rectilinear portions of the log-dose response curves as fitted mathematically from the data in Table 1 for the three experiments using corti-

TABLE 2. SLOPE VALUES AND STATISTICAL DATA OF LOG-DOSE RESPONSE LINES OF THYMUS INVOLUTION RESPONSE TO GRADED DOSES OF VARIOUS STEROID HORMONES

Compound	Lines with slopes b				Lines with slope b_c		
	b	S.D.L.	S.E.$_b$	λ	S.D.L.$_c$	S.E.$_{b_c}$	λ_c
E_1	−121.20	48.92	19.55	.404			
E_2	−144.59	35.14	14.22	.243			
E_3	−133.34	40.74	15.93	.305			
$(E_1+E_2)E_c$	−110.44	42.36	9.78	.318	39.35	9.08	.285
A	−110.44	41.84	15.21	.379	5.06	1.85	.037
F	−151.05	24.93	9.18	.165	44.49	16.39	.294
B_1	−136.82	65.81	24.01	.481			
B_2	−142.94	33.70	11.41	.236			
$(B_1+B_2)B_c$	−140.11	50.74	12.47	.362	54.83	13.60	.407
DCG	32.58	55.64	18.73	1.71			
b_c*	−134.59		7.53	.396			

$b = \frac{\Sigma y(X-\bar{X})}{\Sigma(X-\bar{X})^2}$ = slope of line

$\text{S.D.L.} = \sqrt{\frac{\Sigma(y-y\ \text{calc})^2}{N-2}}$ = Standard deviation about the line.

$\text{S.E.}_b = \frac{\text{S.D.L.}}{\sqrt{\Sigma(X-\bar{X})^2}}$ = Standard error of the slope.

$\lambda = \frac{\text{S.D.L.}}{b}$ = Index of precision

* Slope of lines E_c, A, F, B_c made parallel.

sone as the test compound are presented in Figure 1 (broken lines E_1, E_2, E_3). The relative weights of the thymi are represented on the y-axis and the log-dose of cortisone on the X-axis. The shaded bands represent the means and standard errors of the intact and adrenalectomized controls. Since the slopes have negative values, the equation of the lines is $y = a - bX$, and the values for the slopes of each line are given in Figure 1 and in Table 1.

The differences between the regression constants a and b were not significant and therefore, the three lines were combined. The resultant of the combined lines is represented by the solid line E_c which has a slope of -133.03 ± 9.78. Thus, within the limits of the dosage range (25 to 500 μg /day), for each 10-fold increase in the dose of Cortisone, the weight of the thymus decreased by 133.03 ± 9.78 mg./100 gm. initial body weight. Line E_c also shows that a daily dose of cortisone of between 25 and 50 μg./day/ 18 gm. body weight of adrenalectomized mouse may be expected to maintain the weight of the thymus of the adrenalectomized animal at the level of the intact control.

The response of the thymi to graded doses of Compounds A, B and F showed the same types of inverse rectilinear relationships between the relative weights of the thymi and the log doses of hormones within the same dose range as observed with cortisone. Comparison of the fitted lines

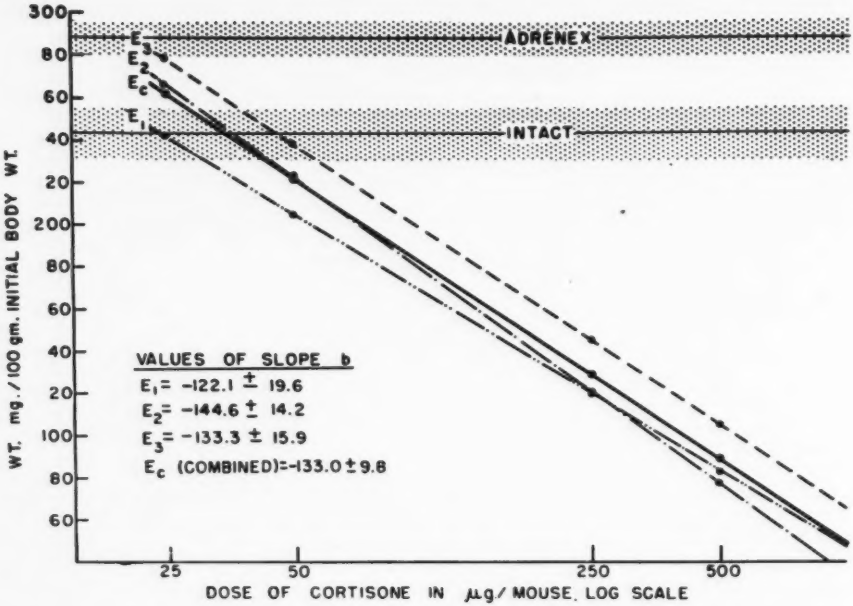


FIG. 1. Log dose-response relationship between the weight of the thymus and graded doses of cortisone. Shaded bands represent means and standard errors of means of control groups.

obtained for these three compounds showed significant differences in the positions of the lines (related to differences in potency) but no significant differences between the values for the slopes *b* (related to the mode of action). Thus, the lines E_c, A, B_e and F were made parallel and given the common slope of -134.59 ± 7.53 (Fig. 2). Within the limits of the dose range, for every ten-fold increase in the dose of any one of the represented hormones, the relative weight of the thymus decreased by 134.59 ± 7.53 mg./100 gm. initial body weight. Also, from Figure 2, the daily dose of each hormone which may be expected to maintain the weight of the thymus in the adrenalectomized animals at the level of the intact controls may be estimated.

From these data, the ratios of potencies between the four compounds may be graphically estimated or mathematically computed (Emmens, 1948). Computation of these ratios show that Compound F is 5.72 times as effective as A, 4.12 times as effective as B and 2.86 times as effective as E in inducing involution of the thymus in adrenalectomized mice. The ratios of potency fall within the 95% limits of confidence. The upper and lower limits are shown in Figure 2.

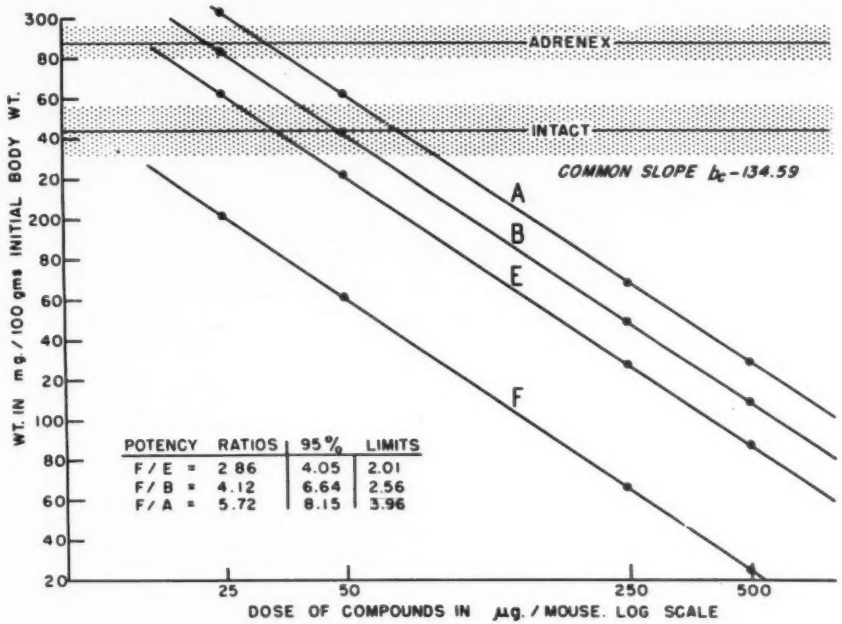

ADRENEX
INTACT
COMMON SLOPE b_c = 134.59
A
B
E
F
WT. IN mg. / 100 gms INITIAL BODY WT.
DOSE OF COMPOUNDS IN µg. / MOUSE. LOG SCALE
POTENCY RATIOS | 95% LIMITS
F / E = 2.86 | 4.05 | 2.01
F / B = 4.12 | 6.64 | 2.56
F / A = 5.72 | 8.15 | 3.96

FIG. 2. Log dose-response relationships between the weight of the thymus and graded doses of Compounds F (hydrocortisone), E (cortisone), B (corticosterone), A (11-dehydrocortisone). Shaded bands represent means and standard errors of means of control groups.

Substances L and S failed to cause any significant change in the mean weights of the thymi as compared to those of the intact and adrenalectomized controls (Table 1). The dose of Substance S was increased to a maximum of 1 mg. but no involutionary activity was found (Table 1). Administration of DCG resulted in a rectilinear response line with a positive slope of 32.58 ± 18.73 in the dose range of 5 to 250 μg. Thus, DCG, within this dose range, caused a small suggestive but not statistically significant ($P < 1.0$) growth of the thymus.

Peripheral lymph nodes

Tables 3 and 4 present the same information for the relative weights of the peripheral lymph nodes as that presented for the thymi in Tables 1 and 2. Of special interest is the fact that the difference between the mean relative weights of the intact and adrenalectomized controls was not significant. The data are further analyzed in Figure 3, which depicts the rectilinear log-dose relationships which exist between the relative weights and

TABLE 3. MEANS OF RELATIVE WEIGHTS* OF PERIPHERAL LYMPH NODES OF ADRENALECTOMIZED MICE TREATED WITH GRADED DOSES OF VARIOUS STEROID HORMONES

Compound	Control non-treated		Adrenalectomized-hormone treated dose in μg./day‡						
	Intact	Adrenalectomized	5	25	50	250	500	750	1000
E_1	73.2	83.9	77.6	72.6	63.8	67.4	57.0		
E_2	134.0	131.9	141.7	126.8	134.3	105.8	75.1		
E_3	130.3	153.1	127.7	148.5	124.5	116.5	95.4		
B_1	127.8‡	116.6†	114.8	124.7	130.3	88.1	102.1		
B_2	94.0	94.9	127.2	104.6	100.7	90.8	85.3		
S_1	116.0	141.7	162.4	146.2	171.8	174.1	202.1		
S_2	119.2	129.6	—	—	114.5	120.3	135.5	115.4	120.7
F	130.1	140.6	154.6	130.8	126.4	74.0	50.0		
L	166.0†	124.0†	163.0	144.5	146.1	158.7	162.0		
A	105.8	133.9	116.4	134.5	117.9	104.1	109.4		
DCG	117.7	168.3	130.4	133.2	137.0	157.1	166.5		
Means of control groups	113.2	130.9							
S.E.	±6.8	±8.8							
P value§	<0.2								

* Mg./100 gm. initial body weight.
† Data not used.
‡ Treated for four days after adrenalectomy.
§ Differences between means of intact and adrenalectomized non-treated controls.

graded doses of hormones with the same compounds and within the same dose ranges as those observed for the thymi. In contrast to the failure of substance L to cause any changes in the weight of the thymus, administration of this hormone resulted in a log-dose response line with a positive slope of 14.4 ± 11.1 for the peripheral lymph nodes. This increase, however, was not significant ($P < 0.2$). The slope of 26.3 ± 8.1 obtained for DCG was significant ($P < .01$). The differences between the positions of line B as compared to that of lines A and E, were significant but that between lines A and E was not. The slopes of the lines E_c, A and B_c were significant as compared to the value of the intact controls, but they were not significantly different from each other. However, the slope for the line F was significantly different from the slopes for the lines B_c, E_c, and A. Thus, lines B_c, E_c, and A were made parallel for the estimation of the ratios of

TABLE 4. SLOPE VALUES AND STATISTICAL DATA OF LOG-DOSE RESPONSE LINES OF PERIPHERAL NODE INVOLUTION RESPONSE TO GRADED DOSES OF VARIOUS STEROID HORMONES

Compound	Lines with slopes b					Lines with slope b_c		
	b	S.D.L.	S.E.$_b$	P†	λ	S.D.L.$_c$	S.E.$_{b_c}$	λ_c
E_1	− 7.96	12.55	5.08	<0.1	1.577			
E_2	−38.87	27.49	11.13	<0.01	.707			
E_3	−34.24	19.84	7.78	<0.001	.579			
$(E_2+E_3)E_c$	−36.47	23.84	6.69	<0.001	.654	27.39	7.69	1.047
B_1	−32.31	18.40	6.71	<0.01	.569			
B_2	−14.68	17.15	5.81	<0.01	1.168			
$(B_1+B_2)B_c$	−22.84	19.82	4.45	<0.001	.868	18.61	4.18	.711
F	−77.35	17.97	7.27	<0.001	.232			
A	−15.99	6.48	2.36	<0.001	.405	9.32	3.40	.356
L	14.44	32.99	11.10	<0.2	2.285			
DCG	26.30	22.24	8.06	<0.01	.846			
b_c*	−26.16		4.18					

* Slope of lines E_c, A, B_c made parallel.
† P Values of slopes (from Students table of T).

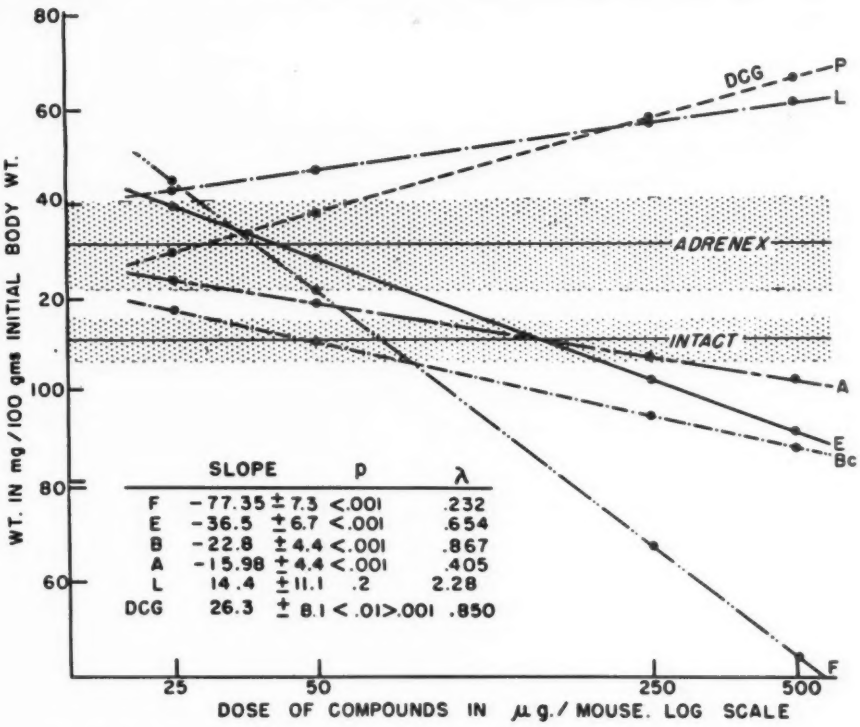


FIG. 3. Log dose-response relationships between the weight of the peripheral lymph nodes and graded doses of Compounds F (hydrocortisone), E (cortisone), B (corticosterone), A (11-dehydrocortisone), Substance L and DCG. Shaded bands represent means and standard errors of means of control groups.

potency of the represented compounds. From the parallel lines (Fig. 4), it may be seen that Compounds E (Cortisone) and A were equally potent and it was computed that Compound B was 3.28 times as effective as cortisone with upper and lower 95% limits of 7.81 and 1.38. Thus, the response of the peripheral lymph nodes to the effect of these hormones, although similar to that of the thymi differs quantitatively as well as qualitatively, i.e. the slopes of the regression lines were smaller and the order of effectiveness was F, B, A, E in contrast to F, E, B, A obtained for the thymus. The peripheral lymph nodes are thus less sensitive to the effects of these hormones and are not practical for this method of analysis. This is attested by the high values of λ for all response lines except for that of Compound F (Table 4, Fig. 4), and the high values of λ_c for the response lines of Compounds B, A and E (Table 4).

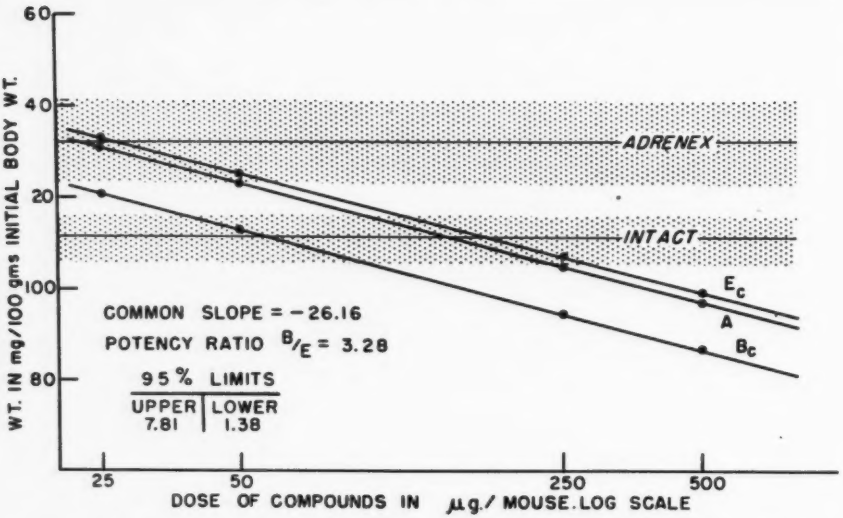


FIG. 4. Log dose-response relationships between the weight of the peripheral lymph nodes and graded doses of Compounds B (corticosterone), A (11-dehydrocortisone) and E (cortisone). Regression lines fitted to a common slope. Shaded bands represent means and standard errors of means of control groups.

Mesenteric lymph nodes and spleens

The results obtained for the mesenteric lymph nodes and spleens were, in general, of the same nature as those obtained for the thymi and peripheral lymph nodes. The variations in weights were so great, however, that it was obvious that they would not be suitable for statistical evaluation.

Histological and cytological changes

A more complete description of the effects of the various hormones used in these investigations will be presented elsewhere. Attention is paid here to a comparison of the changes produced by cortisone and hydrocortisone.

Hydrocortisone is a more effective lymphocytolytic compound than cortisone (Figs. 5, 6) and, also, produces much more marked pycnosis of lymphocytes. Hydrocortisone also tends to produce degenerative changes among the immature lymphocytes of the lymph nodes to a much greater extent than cortisone. Inhibition of homoplastic lymphopoiesis did not appear to be as extensive among the cortisone-treated as among the hydrocortisone-treated adrenalectomized mice, although this observation must be subjected to further quantitative study.

DISCUSSION

It has been reported by numerous investigators that ablation of the gonads and/or adrenals results in an increment in the weight of lymphatic tissue (reviewed by Dougherty, 1952). Further analysis of the change of lymphatic organ weight following adrenalectomy revealed that the lymphatic organs undergo an increase following adrenalectomy, which becomes maximal on the fifth day following operation and then remains relatively constant during the remaining life-span of the adrenalectomized animal (Santisteban, 1951). If, however, adrenalectomized animals are subjected to stress, the growth of lymphatic organs is greater than that due to adrenalectomy alone (Dougherty and Kumagai, 1951). Santisteban, (1951), found that the time following adrenalectomy at which the lymphatic tissue mass becomes maximal and then plateaus remains constant at any age following ablation of the adrenals from the pre-pubertal through the senile period. However, the actual increase is greater in pre-pubertal and senile animals than in those that are sexually mature.

It was first pointed out by Stoerk (1946) that the ratio of thymus weight to body weight of gonadectomized or adrenalectomized rats was not significantly different from that found in well nourished control animals, which grew at an optimum rate. These results were later confirmed by Dougherty and Woodbury (1949) and Santisteban (1951) for mice. When the possibility of adrenocortical-mediated lymphatic tissue involution is eliminated by adrenalectomy, heterogenous stress factors cannot exert their hormonally mediated influence and the values for the lymphatic tissue weights of adrenalectomized animals merely tend to be equivalent to those of the least stressed of intact animals. In order, then, to evaluate the roles of hormones or other agents which may be concerned with the growth of lymphatic tissue, as measured by lymphatic organ weight, it is necessary to establish maximal values which must be exceeded in order to demonstrate the occurrence of true hyperplasia. True hyperplasia has been defined elsewhere (Dougherty, 1952) as the occurrence of a lymphatic organ mass which is greater than that found in animals in which one or all growth moderating influences are not operating. Therefore, true hyperplasia, as measured by the increase in weight of lymphatic organs, occurs when the weights of these organs are greater than those characteristic for the weights at time of maximal increase following adrenalectomy.

Accordingly, the investigations reported here were so designed that the growth promoting effects of the various steroid hormones studied were assayed by giving these hormones during the period of growth of lymphatic tissue which follows adrenalectomy and measurements of lymphatic or-

gans were made at the time of maximal increase following ablation of the adrenals. Among the hormones investigated here, desoxycorticosterone-glucoside produced a significant increase in the weight of the peripheral nodes and a suggestive but not significant growth in the thymus. Compound L, reported elsewhere to increase the weight of the peripheral nodes (Money *et al.*, 1952), produced a slight but not statistically significant increase in the peripheral nodes and had no effect whatsoever on the thymus.

The interrelationship between hormone concentration and degree of involution of lymphatic organs was also studied by administering graded amounts of hormones to adrenalectomized animals. As is the case in evaluating lymphatic tissue growth, the adrenalectomized animal provides a means for a more accurate determination of the quantitative interrelationship of hormone level and lymphatic organ involution. Adrenalectomy eliminates the animals' own hormone supply, thus eliminating the effects of numerous non-specific variables; such as diet, season, alterations in temperature, etc. This is particularly significant since it has been shown that hormone suspending agents within themselves act as non-specific stressors which, in turn, may bring about acute adrenocortical lymphatic tissue involution (Schneebeli *et al.*, 1951).

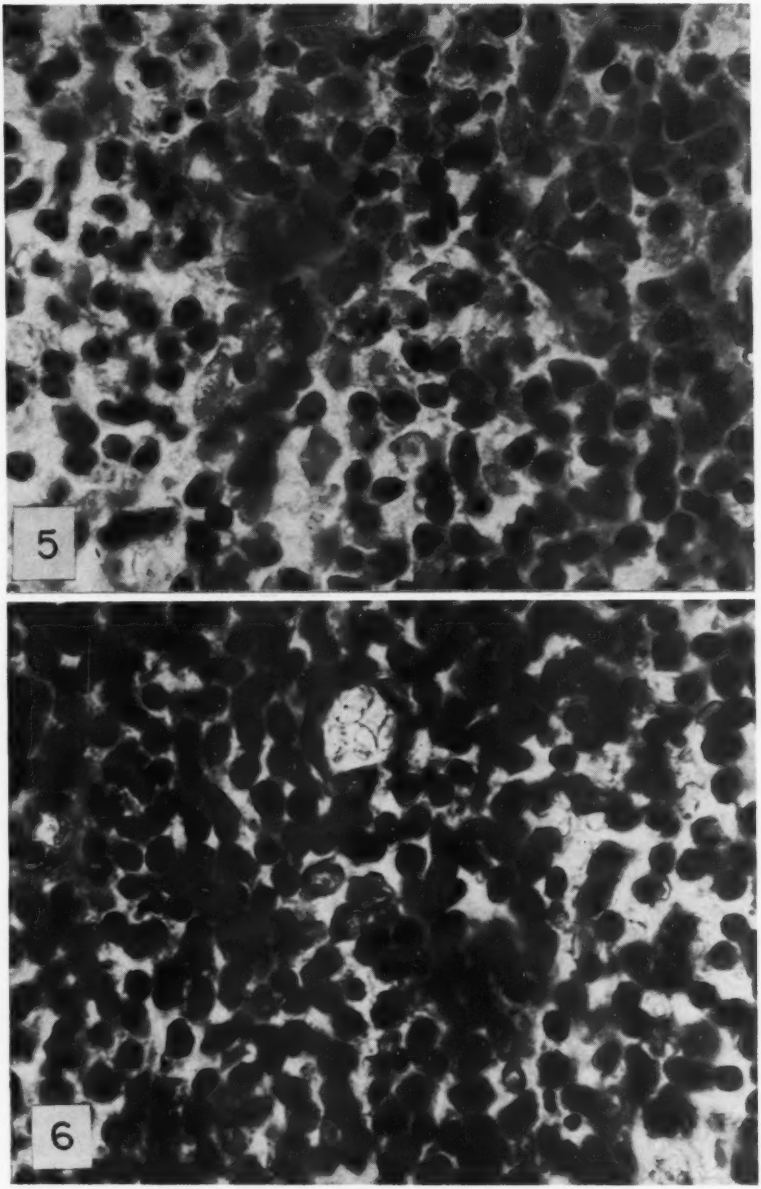
Although gonadal hormones provide a moderating influence on lymphatic organ growth, it did not seem necessary to gonadectomize as well as adrenalectomize, since it is well established that stress effects on lymphatic organs are not mediated by increased secretion of the gonadal hormones (Dougherty, 1952).

The acute involution of the thymus (thymo-regressive effect) produced by the adrenocortical hormones studied, was found to be a response of the continuous variate type. The mathematical analysis of the thymo-regressive curves indicates that there is an excellent correlation between hormone level and thymus size. This conclusion is derived from the reproducibility of the results as demonstrated in the three assays for Compound E, and

EXPLANATION OF FIGURES 5–6

FIG. 5. Inguinal lymph node of adrenalectomized CBA mouse three hours after operation and administration of 0.25 mg. of cortisone acetate. Note the large number of normal immature lymphocytes and reticulo-endothelial cells as compared to Figure 6. The degree of pycnosis is far less than that observed in Figure 6. Section 5μ and stained with methyl green pyronin. Approx. 850×.

FIG. 6. Inguinal lymph node of adrenalectomized CBA mouse three hours after operation and administration of 0.25 mg. of hydrocortisone acetate. The reticuloendothelial cells appear to be undamaged but there are far fewer immature (reticular lymphocytes) than in Figure 5. The extent of hyperchromasia and pycnosis is much greater than that observed following cortisone treatment. Section 5μ and stained with methyl green pyronin. Approx. 850×.



FIGURES 5 AND 6

by the high precision obtained for each of the separate analyses. The parallelism of the lines for the effects of Compounds F, A, B and E on the thymus indicates that the modes of action of these compounds are similar. However, the position of the regression lines showed that these hormones have a different degree of activity on the thymus. These differences in degrees of activity can be mathematically calculated with sufficient accuracy, so that the relative potencies of the thymo-regressive effects can be established with high limits of confidence. The thymo-regressive potencies of the corticoids tested indicate that Compound F is 5.72 times as effective as A, 4.12 as effective as B and 2.80 times as effective as E. From these data, some knowledge of structure activity relationships may be obtained using the structure of Compound F as the molecule of reference. It is evident that an oxy or hydroxy group at position 11 is essential for thymo-regressive activity. An hydroxy group at position 17 enhances thymo-regressive activity and when both C-11 and C-17 have hydroxy groups the greatest effectiveness is observed. The hydroxy group at C-17, however, is not essential for thymo-regressive activity, since both corticosterone and 11-dehydrocorticosterone possessed thymo-regressive action. The necessity for oxy or hydroxy groups at position 11 is demonstrated by the lack of thymo-regressive effect of Substance S.

This method of analysis also allows an estimation of the ranges of the amounts of the different hormones required to maintain different lymphatic organ weights within the range characteristically found in non-stressed intact animals. This may be done by observing the points of intersection of the regression lines with the mean in the normal range of thymus weight of the intact controls. Thus, the daily requirements of each hormone per mouse (18 gm. body weight) for maintaining thymus weight are as follows: Compound F, less than 10 μg.; Compound E, between 25 and 50 μg.; Compound B, between 40 and 60 μg.; and for Compound A it is between 55 and 85 μg. The range of cortisone dosages compares favorably with that amount found by Kumagai (1950) to be effective in preventing post-adrenalectomy increase in weight of lymphatic tissue when given for a period of nine days.

The data obtained using changes of lymph node weights were similar to but differed in certain respects from those found for changes of thymic weights. The peripheral nodes as judged by alterations in weight were not as sensitive to changes in the amount of the administered adrenocortical hormones. Thus, the slopes of the regression lines for all the effective compounds were less than those for the regression lines of changes in thymus weight. This was true even in the case of Compound F where the slope of regression for the peripheral nodes was -77.35 in contrast to the slope of -151.05 for thymo-regression. The standard deviations of the

lines for lymph node regression indicate that the responses of these lymphatic organs are not more variable than those for the thymi; thus values of λ for lymph node regressions are high because the responsiveness of these structures is less than that of the thymi.

The highly statistically significant differences between the slopes of Compound F and the slopes of the lines for Compounds B, E and A clearly indicate that Compound F has a different mode of action on the peripheral nodes than the other three hormones. The order of effectiveness of these steroids on the thymo-regression was F, E, B and A in contrast to the order of effectiveness on the peripheral nodes, which was F, B, A and E. Since the difference in position between lines A and E was not statistically significant, Compounds A and E were equally effective. However, it is important to observe that the slope of the regression line for Compound E, although significantly different from that of Compound F, had a value intermediate between that of F and those of Compounds B and A. This suggests that the mode of action of this compound, may be more similar to that of Compound F than that of Compounds B or A.

It is interesting to note that although Compound L did not cause any change in the weights of the thymi, it did cause a small although statistically not significant growth of the peripheral nodes. Also, desoxycorticosterone glucoside caused a growth of peripheral nodes resulting in a regression line with a slope of $+26.3$, and caused a growth of the thymi resulting in a line with a slope of $+32.58$, which although statistically not significant, had a P value of $<.1$. Thus, the possibility still remains that this compound may prove upon further analysis to possess a true growth promoting effect.

The order of effectiveness of the compounds tested for production of involution of lymph nodes would appear to indicate that the structure activity relationships for lymph node regression are somewhat different than those found for thymo-regression, since Compounds B and A were more effective than cortisone. It should be emphasized, however, that the differences among Compounds A, B and E are relatively small, and these compounds have similar orders of effectiveness in inducing involution of lymph nodes. It is noteworthy that Compound F not only displayed far greater activity than these compounds, but also that the precision for lymph node involution was high and compared favorably with those obtained for thymo-regression. The greater potency of Compound F and its greater precision set the action of this hormone apart from those activities of the other compounds tested.

If the involutionary effects of adrenocortical hormones on thymus and lymph nodes are compared it may be seen that the differences in potency among Compounds A, B and E, as compared to F, are relatively small for both types of lymphatic organs. Thus, the greater effectiveness

of Compound F in inducing acute involution of both thymus and lymph nodes indicates that this hormone has a unique involutionary influence on lymphatic tissue.

There are apparently three mechanisms by which adrenocortical hormones induce acute lymphatic organ involution. They may produce destruction of lymphocytes (lymphocytolysis) and they may also inhibit homoplastic (mitosis) and heteroplastic (differentiation) proliferation of these cells. The relative importance of these hormonal mechanisms in inducing acute involution of lymphatic organs has been discussed in detail elsewhere (Dougherty, 1952). It is apparent from the results obtained by single administrations and prolonged treatment with cortisone and hydrocortisone that lymphocytes in different stages of development display differential susceptibilities to the lymphocytolytic and mitotic suppressing effects of these two hormones (Dougherty, 1953). The small typical mature lymphocyte which is most characteristic of the cells of the thymus is more susceptible to the lymphocytolytic action of the adrenocortical steroids, whereas the immature lymphocytes which are rarely found in the thymus and are characteristically present in lymph nodes are more resistant to this particular hormonal effect (Dougherty and White, 1945). However, immature lymphocytes (reticular lymphocytes) are also diminished in number providing sufficient amounts of hormones are administered.

Since acute lymphatic organ involution occurs as a result of the three mechanisms mentioned above, it is probable that the greater degree of involution of the thymus as compared to that of lymph nodes per unit of administered hormone is due to the fact that the small lymphocyte which constitutes the chief cell type in the thymic cortex is so highly susceptible to the lymphocytolytic action of the adrenocortical steroids. The thymus also is an important center of extremely rapid rate of homoplastic lymphocytopoiesis (Andreasen, 1943), and it has been demonstrated that stress decreases the number of mitotic figures among the lymphocytes of the thymus in intact but not in hypophysectomized or adrenalectomized animals (Pfeiffer *et al.*, 1952). The reduction of mitotic index in stressed animals is proportional to the degree of stress. The parallelism of the regression lines for the different thymo-regressive compounds may be due to the fact that these hormones have in common the qualitative capacities to produce lymphocytolysis and suppress homoplastic lymphocytopoiesis although they differ quantitatively with respect to these mechanisms.

In contrast to the situation in the thymus in which there are few reticuloendothelial cells and no germinal centers, the lymph node is a primary site of heteroplastic lymphopoiesis. It is evident that since the reticular cells

are more resistant to the lymphocytolytic effects of the adrenocorticosteroids, the capacity for heteroplastic lymphocytopoiesis is not completely diminished. Compound F was found to produce the greatest degree of pycnosis of lymphocytes of all the hormones reported in the present study. Although further investigation is necessary, it appears that hydrocortisone produced degeneration of immature cells to a greater extent than cortisone. This unique involutionary effect of hydrocortisone on lymph nodes could be due to its greater capacity to inhibit heteroplastic as well as homoplastic lymphocytopoiesis. The intermediate value of the slope of the regression line for cortisone suggests that this compound may exert a qualitatively similar but quantitatively less effect on heteroplastic lymphocytopoiesis than that exerted by hydrocortisone. Detailed investigation of the influences of these adrenocortical hormones on rates of homoplastic and heteroplastic lymphocytopoiesis are in progress and should shed further light on this problem.

SUMMARY

The effects of several adrenocortical steroids on the growth and acute involution of lymphatic organs of adrenalectomized mice were compared by a method which utilizes the facts that the growth or acute involution of lymphatic organs induced by these hormones are responses of the continuous variate type. It was found that the thymus was the most sensitive of lymphatic organs to the involutionary effects of Compounds F, E, B and A. The response lines had highly significant slopes, high precision and were parallel, but differed significantly in position. The estimated ratios of potency, as estimated from the thymo-regressive lines, were as follows: $F/E=2.8$, $F/B=4.1$, and $F/A=5.7$. The involutionary responses of peripheral lymph nodes (inguinal and axillary) to these same compounds were qualitatively similar, but the slopes of the regression lines were much smaller than those of the regression lines as estimated for thymus involution. The slopes of the lines for Compounds E, B and A were all within range of each other, but that of Compound F was significantly greater.

It is suggested that the observed quantitative differences in the responses of thymi and lymph nodes to involutionary effects of these hormones are correlated with differences in capacities of these substances to influence lymphocytolysis and the processes of homoplastic and heteroplastic lymphocytopoiesis.

Substance S did not induce alterations in weight of any of the lymphatic organs. Substance L produces a small, although not statistically significant growth of the thymus and lymph nodes. A significant growth of both organs was obtained with desoxycorticosterone glucoside.

The structural activity relationships for the growth promoting and involutionary effects of the adrenocortical hormones investigated were established.

The high degree of precision obtained for the lines of thymo-regression suggest that this method of analysis may be used for the bioassay of the effects of pure crystalline hormones on lymphatic tissue and might be adapted to a method for the bioassay of corticoids in body fluids.

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THE COMBINED EFFECTS OF HYPOXIA, ADRENALECTOMY AND THYMECTOMY IN PARABIOTIC RATS

BERNHARD HOELSCHER AND KURT R. REISSMANN[1]

Department of Physiology-Biophysics, USAF School of Aviation Medicine, Randolph Field, Texas

INTRODUCTION

IT HAS been demonstrated by Li and P'An (1950), Houssay and Pinto (1944), Copp and Finerty (1950) that adrenalectomized rats can be maintained in a state of well-being when parabiosed with intact rats. Recently Hoelscher and Reissmann (1952) reported a remarkable difference in heart weight when thymectomized rats joined with adrenalectomized rats which breathed ambient air were exposed to hypoxia. Moreover, it was noted that in several cases the adrenalectomized partner breathing ambient air was survived by the thymectomized partner although exposed to hypoxia.

To offer a more detailed analysis and interpretation of the significance of the previous findings, in particular with respect to the effect on the heart and the nature of transmission through the muscle-skin anastomosis of adrenal steroids, three combinations of intact, adrenalectomized and thymectomized partners of parabiotic pairs were studied both under hypoxia and ground level conditions.

MATERIAL AND METHODS

Rats of the Sprague-Dawley strain maintained on a dog chow ration and given tap water were joined in parabiosis by a muscle-skin anastomosis. After a period from 10 to 12 weeks of parabiotic union, three different types of pairs were established.

Type I—both partners were left intact.
Type II—both adrenals were removed from right partner.
Type III—thymus of the left partner and both adrenals of the right partner were removed.

One week after removal of thymus and adrenals, the pairs were placed in two different categories in both of which all three types were represented. In the first category both partners breathed ambient air; in the second, the right partner breathed ambient air

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[1] Present address: Department of Internal Medicine, Medical Center, University of Kansas, Kansas City, Kansas.

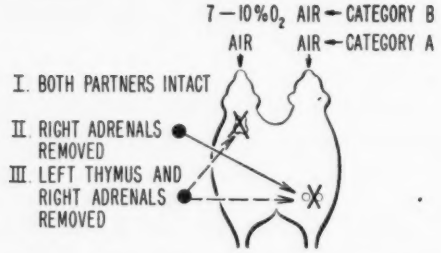
7—10% O_2 AIR — CATEGORY B
AIR
AIR — CATEGORY A
I. BOTH PARTNERS INTACT
II. RIGHT ADRENALS REMOVED
III. LEFT THYMUS AND RIGHT ADRENALS REMOVED

FIG. 1. Diagram showing the three types of parabiotic pairs and the different ways of exposure of the categories A and B.

while the left partner was exposed to an oxygen-nitrogen gas mixture, the oxygen content of which was within a range of 7 to 10 volume per cent. A special breathing chamber consisting of two compartments was employed for exposure. The oxygen content of both compartments was measured with the Beckman electromagnetic oxygen analyzer. The time of exposure was 8 hours a day for five days weekly. The total period of exposure was 4 weeks. Tail blood was used for hematocrit-, hemoglobin-, and red blood cell count studies. The pairs in which the adrenalectomized partner died during exposure were autopsied immediately after death; the others were sacrificed at the end of the desired exposure time of 4 weeks. The dissected organs were weighed to the nearest milligram. In hearts only ventricular weight was determined. Frozen sections were stained with sudan; the paraffin sections with hematoxylin-eosin.

RESULTS

The data obtained on hemoglobin, hematocrit, and red blood cell count are summarized in Table 1.

When both partners lived in ambient air, the blood values obtained for the partners agreed with those observed for single rats. On the other hand, when one partner was exposed to hypoxia while the other partner was breathing ambient air increases in hemoglobin, hematocrit, and red blood cells were found in both partners.

Table 2 shows a summary of the final body, heart, adrenal, and thymus weights. Spleen, kidneys, thyroid and sex organs did not show significant changes in weight and are not included.

Comparing the relative adrenal weights of the different groups, it will be noted that adrenalectomy of one partner led to a compensatory hypertrophy of the adrenals of the co-partner except for group 3. Hypoxia itself did not effect an appreciable increase in adrenal weight in intact partners when united with intact partners as in group 4; however, hypoxia added slightly to a further increase in weight of the adrenals of intact or thymectomized partners when united with unexposed adrenalectomized partners, as in groups 5 and 6.

Histologically, the hypertrophied adrenals produced by adrenalectomy

TABLE 1. AVERAGE HEMOGLOBIN, HEMATOCRIT AND RED BLOOD CELL VALUES FOR THE 2 DIFFERENT CATEGORIES OF PARABIOTIC RATS

Groups	No. of pairs	Type* of pair	Hemoglobin		Hematocrit		Red blood cells—10^6	
			Initial[1]	Terminal[2]	Initial	Terminal	Initial	Terminal
Category A: Both partners living in ambient air								
1	10	I O	15.8± .42†	16.2± .30	43.8± .80	45.1± .67	6.64± .311	7.06± .32
		I O	15.8± .32	16.1± .29	43.8± .76	45.1± .59	6.89± .268	7.23± .286
2	5	I O	15.4± .89	15.7± .86	42.4± .75	43.8± .58	6.55± .389	7.21± .302
		$\bar{A}$ O	15.5± .82	15.8± .60	43.4± .51	43.6± .60	6.82± .559	7.16± .157
3	4	$\bar{T}$ O	15.1± .46	15.2± .37	42.8±1.11	44.8± .85	6.99± .42	7.37± .41
		$\bar{A}$ O	14.8± .74	15.0± .60	42.5± .65	44 ± .91	6.85± .290	7.14± .318
Category B: Partners exposed to different pO_2								
4	4	I $\bar{O}$	15.5± .35	19.0± .55	43.8±1.11	58.0±1.08	6.40± .271	9.14± .363
		I O	15.2± .35	18.8± .26	44.8±1.32	59.0±1.78	6.50± .191	8.99± .387
5	4	I $\bar{O}$	15.7± .45	18.6±1.03	44.2±1.32	58.5±1.44	6.96± .448	9.26± .401
		$\bar{A}$ O	15.4± .30	18.0± .78	43.2±1.32	56.2±2.53	6.73± .457	8.99± .344
6	3	$\bar{T}$ $\bar{O}$	15.2± .59	20.1± .89	43.0±1.53	54.0±1.16	5.92± .982	8.75±1.684
		$\bar{A}$ O	14.7± .58	19.4± .64	43.7±1.20	55.7±1.45	6.13± .843	9.00±1.116

* Each partner is characterized by 2 symbols, the first of which refers to the type of operation, the second to the type of gas mixture.

I = intact, $\bar{A}$ = adrenalectomy, $\bar{T}$ = thymectomy.

O = ambient air, $\bar{O}$ = 7%–10% oxygen-nitrogen gas mixture.

Exposure started 2d week after operations and lasted 4 weeks, 8 hours daily (5 times weekly)

[1] initial = before . . . , [2] terminal = 5 weeks after operations.

† SE (of mean) = $\frac{SD}{\sqrt{n}}$

TABLE 2. AVERAGE BODY, HEART, ADRENAL, AND THYMUS WEIGHTS (GM.) OF 6 DIFFERENT GROUPS OF PARABIOTIC RATS

Groups	No. of pairs	Type* of pair	Final body weight	Heart		Adrenals		Thymus	
				Abs.	%B.W.	Abs.	%B.W.	Abs.	%B.W.
1	16	I O	161±4‡	.563 ±.025	.348 ±.012	.053 ±.000	.033 ±.002	.308 ±.013	.192 ±.009
		I O	171±9	.552 ±.024	.325 ±.008	.056 ±.002	.034 ±.002	.294 ±.020	.174 ±.011
2	7	I O	182±3	.702 ±.034	.387 ±.024	.075 ±.006	.041 ±.004	.211 ±.065	.118 ±.038
		$\bar{A}$ O	235±22	.740 ±.059	.317 ±.012	.045† ±.003	—	.353 ±.032	.157 ±.021
3	7	$\bar{T}$ O	207±15	.771 ±.035	.376 ±.015	.067 ±.006	.034 ±.004	.257† ±.034	— —
		$\bar{A}$ O	214±16	.638 ±.041	.299 ±.006	.048† ±.003	— —	.399 ±.031	.192 ±.020
4	5	I $\bar{O}$	158±2	.561 ±.027	.353 ±.013	.053 ±.003	.033 ±.002	.215 ±.044	.135 ±.027
		I O	190±4	.630 ±.026	.331 ±.014	.060 ±.004	.031 .002	.265 ±.038	.139 ±.020
5	11	I $\bar{O}$	158±9	.679 ±.029	.435 ±.020	.069 ±.006	.045 ±.005	.122 ±.020	.074 ±.010
		$\bar{A}$ O	177±6	.541 ±.027	.306 ±.013	.053† ±.003	— —	.373 ±.032	.210 ±.016
6	11	$\bar{T}$ $\bar{O}$	175±6	.812 ±.056	.471 ±.042	.083 ±.007	.049 ±.005	.363† ±.020	— —
		$\bar{A}$ O	188±7	.515 ±.034	.272 ±.011	.056* ±.004	— —	.352 ±.036	.186 ±.015

* See footnote Table 1.

† Weight of these organs at time of removal.

‡ SE (of mean) = $\frac{SD}{\sqrt{n}}$

of one partner showed a pattern of lipid distribution as observed in normal glands.

A slight reduction in the amount of sudanophilic material of the adrenals was seen in the intact partners which were exposed to hypoxia and joined with intact partners which breathed ambient air, as in group 4. However, a severe loss and clumping of the lipoid globules was encountered in the adrenal cortex of the hypoxic partner when united with an unexposed adrenalectomized parabiont, as in groups 5 and 6.

The thymus was slightly reduced in weight in the hypoxic partners of the intact-intact pairs of group 4. In group 2, the thymus of the intact partner with hypertrophied adrenal cortex was invariably diminished in weight;

TABLE 3. TESTS OF SIGNIFICANCE FOR THE AVERAGE DIFFERENCES OF PERCENT HEART WEIGHTS BETWEEN THE TWO PARTNERS WITHIN EACH GROUP OF THE SIX DIFFERENT GROUPS OF PARABIOTIC RATS

Groups	No. of pairs	Conditions* of partners		Average differences between heart weights of the two partners	Variance estimate of the differences in heart weight of the pairs	Estimate of standard deviation of average differences	t	P†
		Left	Right					
1	16	I	O—I O	.0233	.00318	± .0141	1.653	.124
2	7	I	O—$\overline{A}$ O	.0696	.00175	± .0158	4.405	<.01
3	7	$\overline{T}$	O—$\overline{A}$ O	.0777	.00145	± .0144	5.397	<.01
4	5	I	$\overline{O}$—I O	.0216	.00084	± .0130	1.662	.178
5	11	I	$\overline{O}$—$\overline{A}$ O	.1294	.00731	± .0258	5.016	<.01
6	11	$\overline{T}$	$\overline{O}$—$\overline{A}$ O	.1988	.02049	± .0431	4.613	<.01

* See Footnote Table 1.
† The difference between heart weights was found by subtracting the percent heart weight of the right partner from that of the left partner and was considered statistically significant if P was smaller than .01.

this change was accentuated when such a partner breathed an oxygen deficient gas mixture as was the case in group 5.

In many cases the livers of parabionts exposed to hypoxia and joined with adrenalectomized partners appeared yellowish and showed a slight fatty infiltration microscopically compared with those of adrenalectomized partners which were reddish brown in color and without signs of fat infiltration.

The most striking finding was the difference between the heart weights of the two partners following adrenalectomy of one partner. This difference turned out to be more conspicuous when the non-adrenalectomized partner was exposed to hypoxia. As will be seen from Table 3, a statistically significant difference in percent heart weights between the two partners was found for groups 2 and 3 in which an adrenalectomized partner was parabiosed with an intact or thymectomized partner.

This effect of adrenalectomy of one partner was more pronounced in groups 5 and 6 in which the intact or thymectomized co-partners were exposed to hypoxia.

The adrenalectomized partner breathing ambient air in most instances died first when hypoxia was imposed on the co-partner. Before death the adrenalectomized partners showed signs of apathy, dizziness and at times muscle twitches.

DISCUSSION

It is generally agreed that the rate of formation and release of ACTH from the pituitary gland is determined by the concentration of adrenal cortical hormones in the blood (Ingle *et al.*, 1938; Sayers and Sayers, 1947). According to data reported by Gemzell, Van Dyke, Tobias and Evans (1951) the ACTH plasma level increases steeply after adrenalectomy until it reaches 30 times the normal level three weeks thereafter and remains unchanged. Furthermore, their data suggest an increase in the ACTH plasma level in a non-adrenalectomized partner when united with an adrenalectomized partner, which is usually accompanied by a corresponding increase in size of the adrenals. Comparison of the relative adrenal weights of the parabionts of groups 1 and 2 lends support to their data.

Hypoxia itself did not produce statistically significant changes in adrenal size when both partners were intact as in group 4. It might well be that in these cases the intact unexposed partner released small amounts of cortical hormones to the exposed partner, which prevented an appreciable enlargement of the adrenal cortex of the hypoxic partner. In this connection, however, it should be mentioned that enlargement of the adrenal glands is not always a dependable criterion of its function (Sundstroem and Michaels, 1942; Darrow and Sarason, 1944).

The relative weights of the hearts of the partners of intact-intact parabiotic pairs were usually slightly increased compared with those of single rats as was true also of the adrenals. This seems to be due to the stress of the parabiosis itself. However, after adrenalectomy of one partner there was a considerable difference between the relative heart weights of the two partners. The heart weights of the non-adrenalectomized partners were definitely increased in weight above the normal level, whereas the heart weights of the adrenalectomized partners were below the normal level. An insufficient supply of adrenal cortical hormones of the adrenalectomized partner and an accompanying increased uptake by the co-partner of adrenal cortical hormones from its hypertrophied adrenals obviously cause this difference in heart weights. Analogous clinical findings of small hearts and the readily noticeable increase in heart size following cortical hormone treatment of patients with Addison's disease give supporting evidence to this view. The severe loss and clumping of the lipoid globules found in the glomerulosa and outer fasciculata layers of the adrenal cortex of the hypoxic partner and the premature death of its unexposed adrenalectomized

partner are indications of an adrenal cortical insufficient state which is ultimately reached by the adrenal cortex of the hypoxic partner. An additional exchange of electrolytes between the partners might have a beneficial effect on the condition of adrenal insufficiency as has been shown by Ingle and Oberleve (1946).

The fatty infiltration found in livers of hypoxic parabionts joined with unexposed adrenalectomized partners might be due to metabolic processes which have not been thoroughly investigated as yet. Steroid compounds have been shown to be metabolized for the most part in the liver (Pincus and Pearlman, 1943); furthermore, a fat transfer into the liver under the influence of corticoids has been described by Levin (1949). Since the fatty changes in the liver cells were found only in hypoxic partners, hypoxia appears to be at least one of the main factors directly or indirectly causing such a fat accumulation within the liver cells. Similar findings have been reported by Highman and Altland (1949).

Distinct regression of the thymus was invariably observed in parabionts with enlarged adrenal glands. Whether or not thymectomy modifies the response to ACTH, no unequivocal information has as yet been obtained from these experiments.

The increased hemoglobin, hematocrit and red blood cell values were found in both partners, when one parabiont was exposed to hypoxia. These findings are in agreement with observations of Reissmann (1950) who has discussed this phenomenon more in detail.

SUMMARY

Three combinations of intact, thymectomized and adrenalectomized rats have been studied with the parabiotic technique. Either both partners of the pairs breathed ambient air or one partner was exposed to hypoxia while the other partner breathed ambient air.

Hemoglobin-, hematocrit-, and red blood cell count values were increased in both partners when hypoxia was imposed on one parabiont.

Adrenalectomy of one parabiotic partner was followed by a compensatory hypertrophy of the adrenals left in the co-partner which was higher in degree following exposure to hypoxia.

There was a remarkable difference in heart weight between the two partners when adrenalectomy was carried out in one partner. The heart was smaller in the adrenalectomized partner than in the co-partner. This difference was more conspicuous when the non-adrenalectomized partner breathed an oxygen deficient gas mixture.

The livers of hypoxic partners when united with unexposed adrenalectomized partners showed a slight fatty infiltration.

Removal of the thymus had no appreciable effect.

The adrenalectomized partner breathing ambient air in most instances died first when hypoxia was imposed on the co-partner. This suggests that the hormonal output of hypertrophied adrenals, which meets the hormonal requirements of both partners when breathing ambient air, becomes critically insufficient for the adrenalectomized partner when the demand for adrenal cortical steroids is increased in the opposite member by hypoxia.

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THE RATE OF IODINE METABOLISM BY THE THYROID FOLLICLE AS A FUNCTION OF ITS SIZE[1]

N. J. NADLER,[2] C. P. LEBLOND AND R. BOGOROCH[3]

Department of Anatomy, McGill University, Montreal, Canada

THE thyroid gland is an aggregate of minute follicles. These are spheroids composed of a layer of epithelial cells enclosing a viscous fluid, the colloid. The follicles are independent units—a fact particularly obvious in fish where isolated follicles are scattered over a large area ventral to the pharynx.

While it is generally agreed that the follicle is the functional unit of the thyroid gland, there have been widely different interpretations of its mode of activity. The once popular "reversal theory" assumed that the polarity of the epithelium is periodically reversed, that is, the cells *alternately* secrete colloid into the follicle lumen and resorb it for excretion into the circulation (Thomas, 1934; Williams, 1937). However, the advent of radioautography made it possible to show that all follicles were at all times depositing thyroglobulin, the main component of colloid, into the lumen. This fact disproves the reversal theory (Leblond and Gross, 1948).

Studies carried out on the whole gland indicated that all the stages in the elaboration and release of the thyroid hormone occur "simultaneously, continuously, and at a constant rate" (Dougherty *et al.*, 1951). This is probably true also of individual follicles. However, when the various follicles of a gland are compared, they function at different rates. This is obvious even on superficial examination of a thyroid radioautograph since there are wide variations in the intensity of the radioautographic images of individual follicles.

In the present work, the rates at which individual follicles accumulate (i.e., bind in organic form) and release iodine were determined by a technique referred to as "quantitative radioautography." At various time intervals after administration of radioiodine, the density of the radioautographic images of the follicles was estimated by counting the number of developed silver grains per unit area of overlying photographic emulsion

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[2] Holder of a Fellowship in the Medical Sciences administered by the National Research Council for the Rockefeller Foundation.

[3] Present address: Department of Oral Pathology, Harvard School of Dental Medicine, 188 Longwood Avenue, Boston, Massachusetts.

(Nadler, 1953). From these results, the relative radioiodine concentration in the follicles was calculated as a function of time.

An attempt was then made to relate the variations in the radioiodine concentration of the follicles to their structural characteristics. The results indicated that the rate of activity of any follicle is a function of its size.

METHOD

Thirty-six male albino rats fed on Purina Fox Chow and weighing 110–125 mg. were each injected subcutaneously with 25 μc. of radioiodide. They were subdivided into six groups of 6 animals which were later sacrificed at 1, 4, 10, 50, 100 and 200 hours respectively. The right lobe of the thyroid gland was fixed in Bouin's solution, while the left lobe was digested in 5 cc. of 2 N NaOH, plated in duplicate, and counted by an end-window beta-ray Geiger counter. To permit calculation of the fraction of the injected dose fixed by the gland, aliquots of the injection solution were counted in the same manner. In this and subsequent cases, all figures were corrected for radioactive decay. The variation with time in the thyroid uptake of radioiodide is plotted in Figure 1.

All the thyroids of a given group of animals were embedded in a single block and sectioned at 7μ. Two sets of slides were prepared. Each slide of the first set included sections from the blocks of the 1, 4, 100, and 200-hour groups. The second set consisted of sections from the 4, 10, 50, and 100-hour groups. Therefore, the overlapping groups (4 and 100-hour) could be used to compare the two series.

The slides were stained with Masson's trichrome or left unstained, and then radioautographed by the coating technique (Bélanger and Leblond, 1946) using Ilford halftone emulsion (Gross *et al.*, 1951). Suitable precautions were taken to insure uniform technical procedure: all slides were coated with emulsion at the same time; exposure time was kept short. However, due to the wide range of isotope concentrations, two different exposure times had to be selected: a longer exposure time to study the less radioactive sections of the first set, and a shorter one to compare the more radioactive sections of the second set.

For each time interval, only two thyroids were investigated in detail. (The two thyroids selected were those whose percentage uptake of radioiodine—as determined for one whole lobe as described above—was closest to the average for the six animals sacrificed at that time.) A large scale projection tracing of the thyroid section under investigation was made and individual follicles were drawn. The intensity of the radioautographic images of each follicle was then investigated by counting silver grains at different levels of the emulsion in a 70 square μ area in the center of the colloid (Nadler, 1953). A total of 400–500 follicles was counted for each time interval. In addition, the average diameter of the colloid was measured by means of a micrometer placed in the ocular. The staining property of the colloid was recorded as red, green, or mixed. The position of the follicle near the periphery or the center of the gland was noted.

THEORY

The purpose of this investigation was to evaluate the functional activity of thyroid follicles from the number of silver grains counted in their radioautographic image. The interpretation of these counts involved several theoretical considerations:

1) The grain counts had to be expressed as a function of the amount of impinging radiation (exposure) and, from this, as concentrations of radioiodine in the colloid.
2) The follicles on which the counts had been performed were defined by their size.

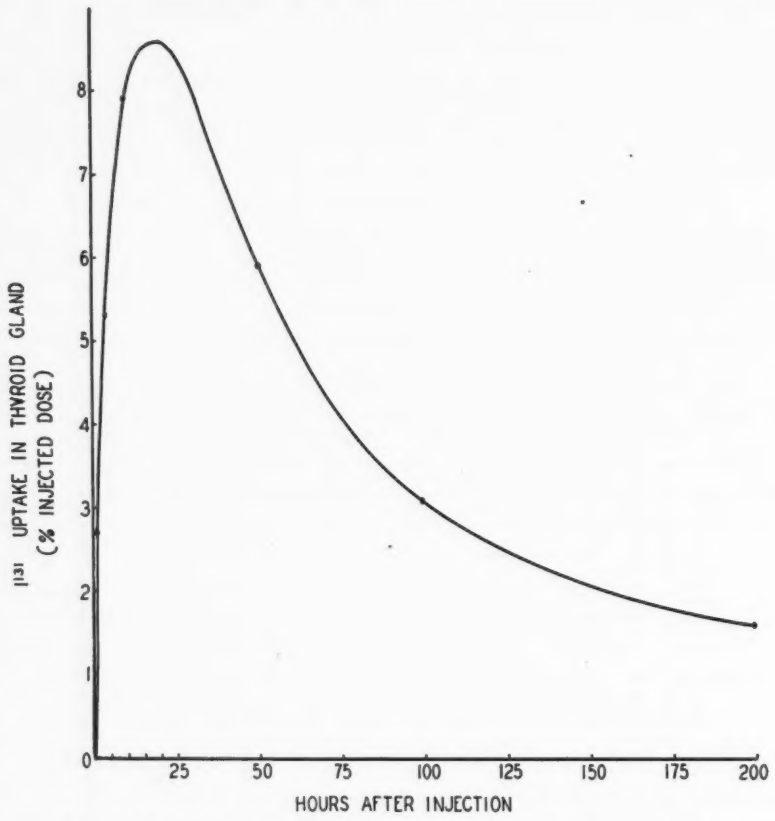


FIG. 1. Uptake of radioiodine by the entire thyroid gland at various time intervals after administration of radioiodine.

This required that the diameters measured in follicle sections (crude diameters) be corrected to yield the true diameters.

3) From these data, the rate of iodine metabolism by follicles of various sizes had to be calculated.

1) Quantitative radioautography of radioiodine in the thyroid follicle

Results summarized elsewhere (Leblond, 1951) indicate that most of the radioiodine appearing in the thyroid is present as thyroglobulin. The newly-formed labeled thyroglobulin generally diffuses in a rapid and even manner throughout the colloid. This is shown by the uniform radioautographic image produced over any follicle in Figures 4–9. Such reactions of the colloids were investigated here.[4]

[4] At early time intervals the radioautographic images may appear as "ring" reactions. Later, these are replaced by reactions which are diffuse throughout the colloid (Leblond and Gross, 1948). Follicular ring reactions were rare in the present series (Figs. 4 and following) and were disregarded.

The theory and methods used to relate the number of grains counted in these radioautographic images to the concentration of radio-element in the sections of thyroid follicle were previously reviewed (Nadler, 1953). It was there shown that the grain count in a radioautographic image is proportional to the amount of incident radiation (exposure) as long as the exposure time is kept short. The relationship between the amount of incident radiation and the concentration of radioactivity in the tissue source depends on the geometric conditions (distance, size, etc.). But, when the tissue source is a histological section of follicle colloid prepared under controlled conditions, the only variable geometrical factor is the diameter of the colloid.

To calculate the effect of such a source, it was assumed that the radiation distributed itself at a distance from the source according to the inverse square law (Nadler, 1951). Experimental evidence indicated that this was partially true; it also supplied the data required for appropriate corrections (Nadler, 1953). Thus, the relative radioiodine concentration in a follicle section could be calculated from the grain count.

2) Determination of the radioiodine concentration as a function of the true diameter of the follicle colloid

For each follicle section in which the grain count was performed, a measurement was made of the diameter of the colloid, and reported as the "crude diameter." Mathematical considerations made it possible to deduce from the frequency of occurrence of various crude diameters what the frequency of follicles with any given true diameter should be

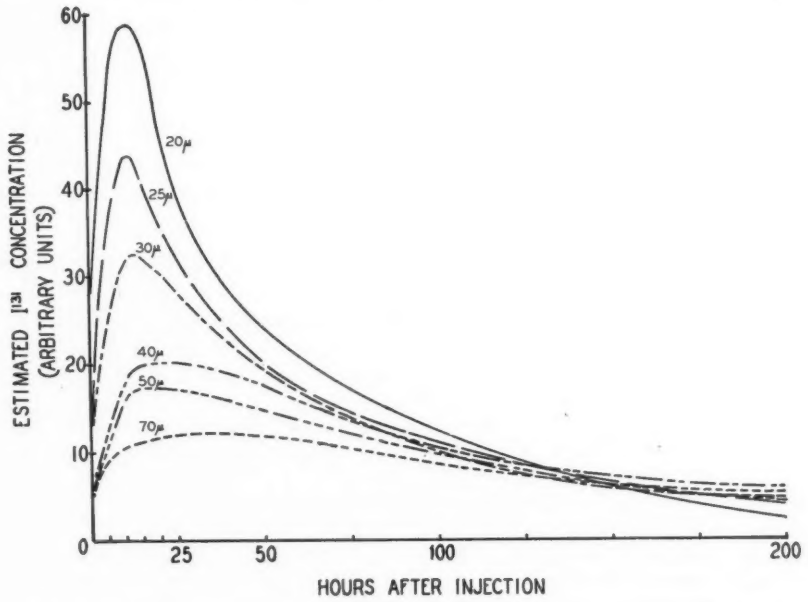


FIG. 2. Variation in concentration of radioiodine with time in various follicle classes identified by the diameters of the colloid measured in microns. It is apparent that, compared to the larger follicles, the smaller ones accumulate the isotope more actively, reach a higher peak concentration, and then discharge the element at a faster rate.

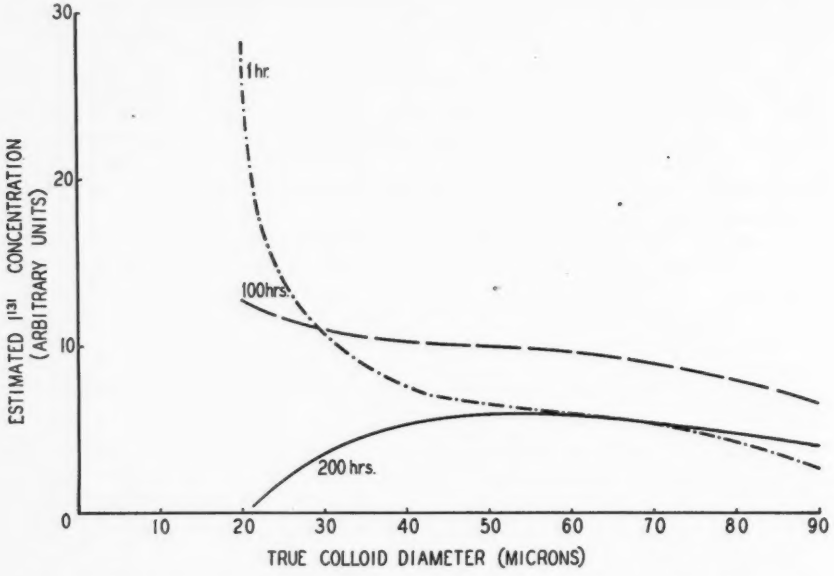


FIG. 3. The concentration of radioiodine in follicles of various colloid diameters reported for three time intervals after the administration of the isotope. The smallest follicles have the highest concentration of radioiodine at the early but not at the late intervals.

(Appendix 1). Calculation of the radioiodine concentration as a function of the true colloid diameter was then possible. Thus, the mean radioiodine concentration in follicles of a given diameter may be obtained (Figs. 2 and 3).

3) Relationship between the radioiodine concentration of the follicle and the rates of iodine fixation and release

Let D be the true colloid diameter of a given follicle, taken to be spherical;

q_i' be the constant rate of accumulation—or organic binding—of iodide per unit surface area of the follicle colloid (so that the amount of iodide organified in the follicle per unit time is $\pi D^2 q_i'$);

q_0' be the constant rate of discharge of organic iodine per unit surface area of the follicle colloid (so that the amount of such iodine leaving per unit time is $\pi D^2 q_0'$);

c represent the concentration of radioiodine in the follicle colloid (so that the amount of radioiodine contained in the colloid is $\pi D^3 c/6$);

x represent the specific activity of the extracellular radioiodide;

y represent the specific activity of the organic iodine material (thyroxine and triiodothyronine) available for release from the follicle into the circulation.

The last three are functions of time, t. The problem examined here is to determine how q_i' and q_0' vary with D.

In any given follicle, the quantity of radioiodine present varies as a result of the

balance between the entry and the exit of labeled iodine—a fact expressed in the following equation:

$$\frac{d}{dt}\left(\frac{\pi D^3 c}{6}\right) = \pi D^2 q_i' x - \pi D^2 q_0' y \tag{1}$$

which may be simplified by making $6q_i' = q_i$ and $6q_0' = q_0$

$$\frac{dc}{dt} = \frac{q_i}{D} x - \frac{q_0}{D} y \,. \tag{2}$$

At very early time intervals, such as 1 hour (and perhaps 4 or 10 hours), a negligible amount of radiothyroxine has left the gland and y may be made equal to 0, giving

$$\frac{dc}{dt} = \frac{q_i}{D} x. \tag{3}$$

This equation is solved as follows:

$$q_i = \frac{Dc}{\int_0^t x dt} \quad \text{(when } t = 0,\ c = 0\text{)}. \tag{4}$$

It is implied that $\int_0^t x dt$ is identical for follicles of any diameter in any location and, therefore, that at a given time, x, the specific activity of the extra-cellular iodide, is the same throughout the gland. Using the value of c determined for follicles of a given diameter, D, it is possible to calculate the relative rates q_i for follicles with this diameter. (The effects of a uniform tissue shrinkage due to histological processing would cancel out in equation 4.)

At late time intervals, x may be made equal to 0, and equation 2 becomes:

$$\frac{dc}{dt} = -\frac{q_0}{D} y. \tag{5}$$

It is known (Riggs, 1952; Fish et al., 1952) that, at these late times, the radioiodine content of the whole thyroid decreases logarithmically with time. Assuming that the same relation holds for the individual follicle, then $(dc/dt) \propto -c$; and, therefore, from equation 5, $y \propto c$. Hence, $y = c/M$; where the factor M is a constant. Since, in the case of the whole gland, a similar factor is proportional to the total iodine concentration (Riggs, 1952; Nadler and Leblond, unpublished), here, M may also be directly related to the iodine concentration of the follicle. Hence,

$$\frac{dc}{dt} = -\frac{q_0}{D}\frac{c}{M}. \tag{6}$$

This equation is solved as follows:

$$q_0 = \frac{DM \ln \frac{c_1}{c_2}}{t_2 - t_1}. \tag{7}$$

Here it is necessary to assume that M is the same for all follicles. By determining the mean concentrations c_1 and c_2 for follicles of a given diameter, D, at times t_1 and t_2 respectively, it is possible to calculate the relationship between q_0 and D (Table 1, last column).

The rate of accumulation, q_i, can also be established from the maximum value of c (Table 1) since it can be shown that under normal conditions, very nearly,

$$q_i \propto D c_{\max}. \tag{8}$$

TABLE 1. RATES OF ACCUMULATION AND DISCHARGE OF IODINE *per unit colloid surface* IN THYROID FOLLICLES OF VARIOUS SIZES

True colloid diameter (microns)	q_i' Accumulation rate				q_0' Discharge rate
	(Arbitrary units: % of mean)				
	Time (hrs.) after I^{131} administration, at which the results obtained were used for calculation				
	1 (eq. 4)	4 (Eq. 4)	10 (Eq. 4)	*$\dot{tm}$ (Eq. 8)	(50–100) (Eq. 7)
90	63	—	—	—	—
70	101	92	87	94	130
50	90	85	95	96	100
40	82	—	88	91	105
35	107	101	—	—	—
30	110	96	108	96	105
25	93	120	122	112	74
20	153	130	—	111	68

* tm = time at which the colloid radioiodine concentration is maximum (derived from Fig. 2).

The figures are averages of determinations, in which the grain count was more than twice background and the number of follicle sections counted was 10 or more. (Otherwise, blank spaces were placed in the table. The follicles with a diameter of 15μ were too few to be reported.)

Assuming that there exists a steady state with respect to the concentration of iodine in the follicle, then $q_i = q_0$ and, therefore, both are related to D by the same mathematical function.

RESULTS

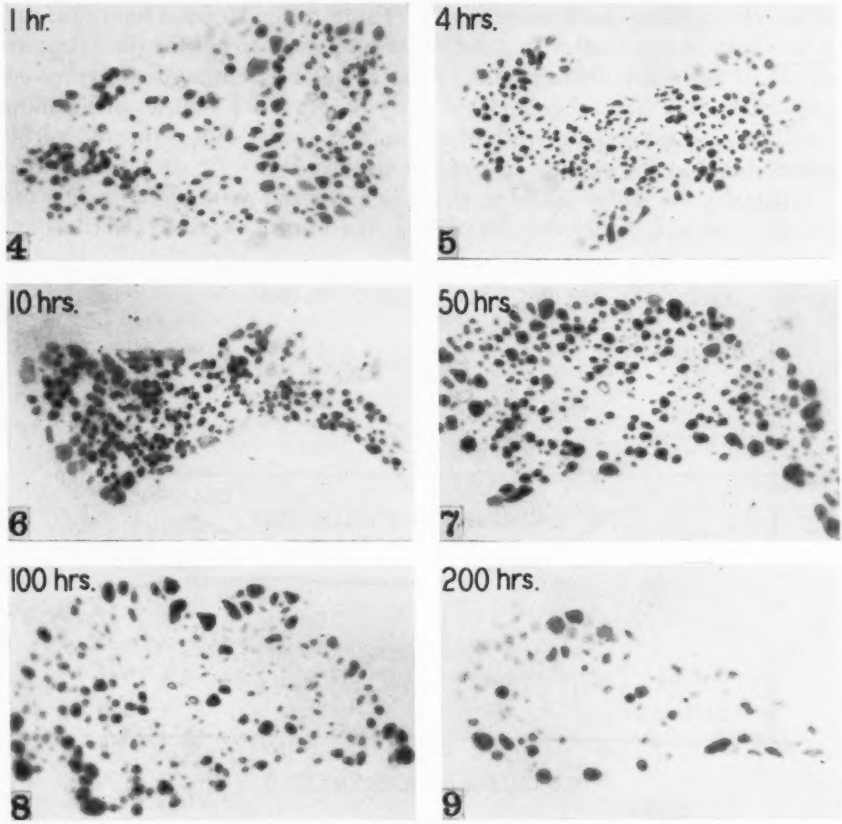
With time, the *radioiodine concentration* of follicles of various sizes increased rapidly to a maximum and then decreased more slowly (Fig. 2, Cf. Fig. 1).

The smaller the follicles, the greater was the peak of radioiodine concentration (Fig. 2). The phenomenon may be visualized in the radioautographs obtained at early intervals after radioiodine injection, when a high concentration of the element was seen in small follicles (usually centrally located in the gland) and a small concentration in large follicles (usually peripherally located) (Figs. 4–6).[5]

The rate of decrease of the radioiodine concentration from the peak values was rapid in the small follicles, but slowed as the follicles increased in size (Fig. 2). As a result, by 200 hours the radioiodine concentration in the larger follicles was about twice that of the smaller follicles. Radioautographs at that time showed a more intense reaction in large than in small follicles (Fig. 9).

These results were confirmed by plotting the radioiodine concentrations

[5] In general, the large peripheral follicles tended to be acidophilic while the smaller central follicles were less or not acidophilic.



FIGS. 4–9. Radioautographs of unstained thyroid sections (40×) at various time intervals after the injection of radioiodine. One of the two thyroids counted at each interval is shown. At one, four and ten hours (Figs. 4, 5, and 6) the smaller follicles give a sharp reaction, but the larger ones are barely visible. By 50 hours (Fig. 7), the reaction intensity has become weak in many small follicles and strong in many large follicles. Later, the reaction of the small follicles decreases further (100 hours, Fig. 8) and almost disappears (200 hours, Fig. 9), while the large follicles are more reactive. In general, the small follicles are central, and the large ones, peripheral.

against colloid diameters (Fig. 3). The curve obtained at 1 hour clearly showed a greater concentration in small than in large follicles, while the curve obtained at 200 hours revealed a reversal of the situation.

The *rate of accumulation of iodide* expressed *per unit surface* area of colloid (that is, q'_i) was calculated for the follicles of various diameters from the results obtained at 1, 4, and 10 hours by means of equation 4. The mean of the values was made equal to 100 and individual results were expressed

as percent of the mean. It may be seen (Table 1; Fig. 10, solid line) that the values vary around 100 within not too wide limits, except for the extremes of follicle size; and, therefore, the rates of iodide accumulation expressed per unit surface area of colloid are fairly constant for follicles of medium size. Thus, in general, the rate of accumulation of iodide appeared to be proportional to the surface area of the colloid.

Similarly, the *rate of discharge of iodine* expressed *per unit surface area of colloid* (that is, q_0'), was calculated from the results obtained for the time

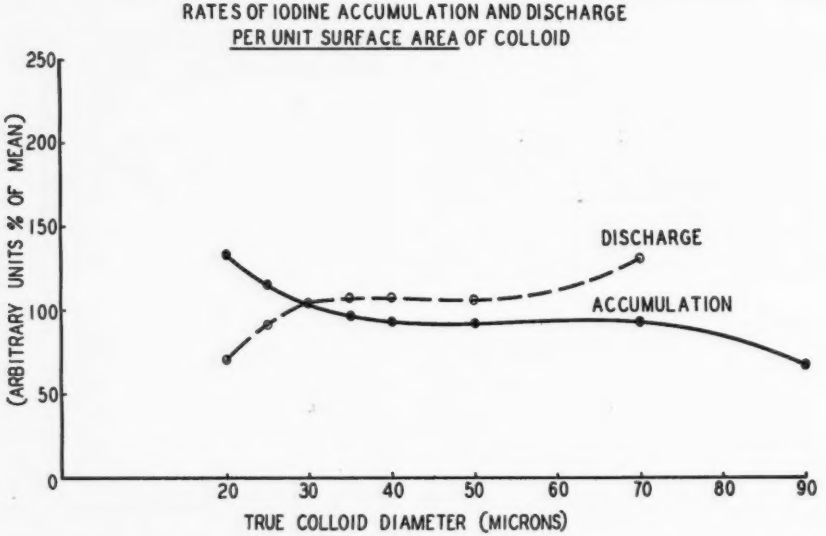


FIG. 10. The rates of iodine accumulation and discharge *per unit surface area of colloid* determined for follicle classes of various colloid diameters.

The values for the accumulation rates, which are taken from column 1 of Table 4, are the weighted means of the determinations at 1, 4, and 10 hours after radioiodine injection (Table 1). The values for the discharge rates are taken from the last column of Table 1.

For follicles in the intermediate class range (colloid diameter 30–60 μ), the rate of iodine accumulation and discharge per unit surface area of colloid is constant.

interval 50–100 hours by means of equation 7 (Table 1, last column). The results were rather uniform in the follicles of intermediate size, that is, those with a colloid diameter of 30–50μ (Fig. 10, broken line), suggesting that the rate of iodine discharge in these follicles is also proportional to the surface area of the colloid.

In addition, these results were expressed as the rate of accumulation of iodide *per follicle* (that is, $\pi D^2 q_i'$) for follicles of various diameters (Fig. 11). As expected, the total amount of iodine taken up by the smaller fol-

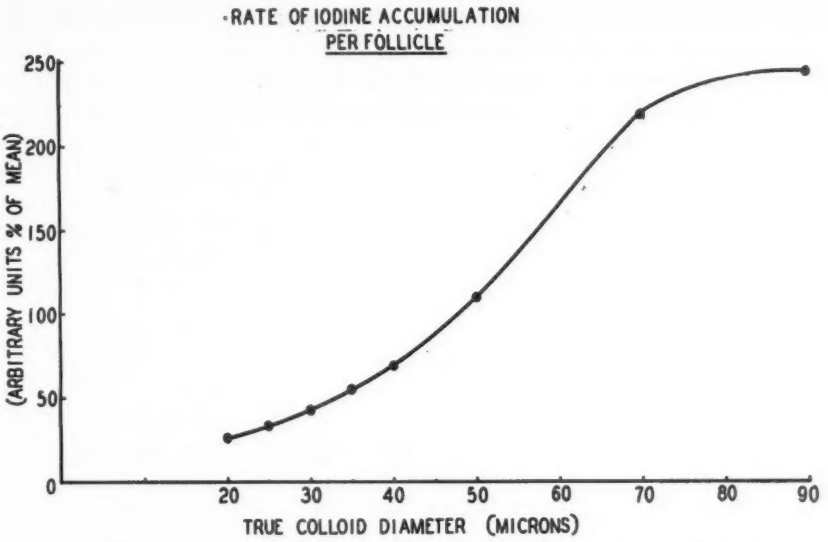


FIG. 11. The rate of iodide accumulation *per follicle* determined for follicle classes of various colloid diameters. It is clear that the smaller follicles accumulate less iodine than the larger ones.

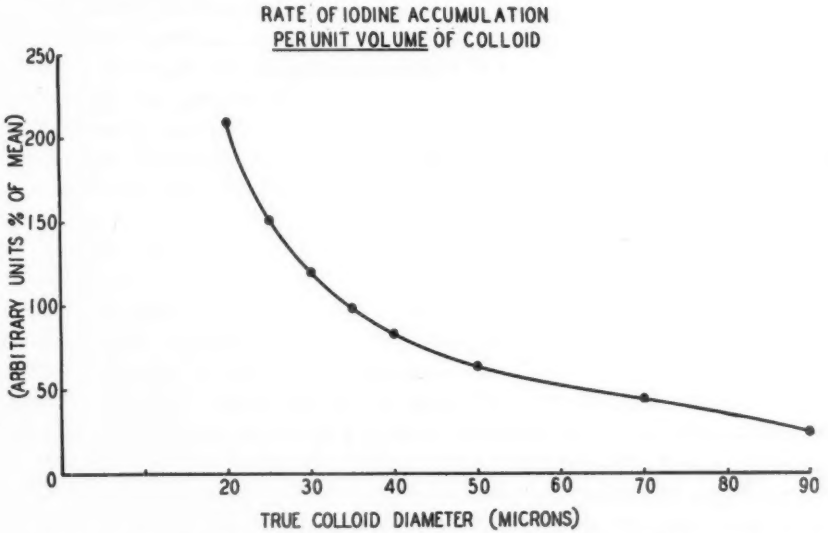


FIG. 12. The rate of iodide accumulation *per unit volume of colloid* determined for follicle classes of various colloid diameters. Small follicles show higher rates than large follicles.

licles was less than that taken up by the larger ones. This rate was also calculated *per unit colloid volume* (that is, $6q_i'/D$), in order to measure the efficiency of follicles of various diameters with regard to their ability to concentrate iodine (Fig. 12). The efficiency was thus found to be greater in the smaller than in the larger follicles.

TABLE 2. BIOLOGICAL HALF-LIFE OF ORGANIC IODINE IN THYROID FOLLICLES OF VARIOUS SIZES

True colloid diameter (microns)	Days
70	4.9
50	3.9
40	2.9
30	2.2
25	2.6
20	2.3

Finally, the *biological half-lives* of the colloid iodine for follicles of various diameters (Table 2) were obtained by application of equation A3 (see Appendix 2). The turnover of iodine was thus found to be faster in small than in large follicles.

DISCUSSION

The thyroid follicles of a given gland differed in their structural characteristics. In the young adult rats under investigation here, the size of the follicles varied to such an extent that the range in the true diameters of the colloid extended from 15 to 100 μ or more, and thus the volume of the largest follicles was more than 300 times greater than that of the smallest ones. The main finding in this investigation was that these differences in follicle size were associated with, and presumably responsible for, functional differences; the smaller the follicles, the more actively they metabolized iodine.

Validity of the results

The amount of radioiodine present in the colloid at different time intervals was estimated by quantitative radioautography. This method assumed that the density of radioautographic images is directly proportional to the amount of incident radiation for any low exposure. The validity of this assumption was tested by counting grains in two radioautographs from the same thyroid exposed for two different periods of time, 11 and 27 hours. The radioiodine concentrations based on the grains counted in these two radioautographs were found to be in approximately the same ratio for each follicle class, that is, about 2.2 (Table 3). Theoretical calculations showed that the ratio of the amounts of radiation delivered during these two periods was 2.4 (Appendix 3). This small discrepancy (10%) between

the experimental and theoretical figures is satisfactorily explained in that the 27-hour exposure yielded grain densities which were at the upper limit compatible with counting. For these high densities, the predicted deviation in the experimental value is also 10% (Nadler, 1953). Thus, it was concluded that the grain counts gave results in keeping with the theoretical expectation for lower densities.

TABLE 3. RELATIVE RADIOIODINE CONCENTRATIONS IN THYROID FOLLICLES OF VARIOUS SIZES ESTIMATED IN RADIOAUTOGRAPHS PREPARED FROM THE SAME THYROID LOBE BUT EXPOSED FOR TWO DIFFERENT LENGTHS OF TIME

True colloid diameter (microns)	Relative radioiodine concentration (arbitrary units)		Ratio of concentrations at the two time intervals
	Exposure time		
	11 hrs.	27 hrs.	
70	4.4	9.7	2.2
50	5.9	12.3	2.1
35	9.4	20.9	2.2
30	11.8	23.6	2.0
25	17.9	36.3	2.0
20	26.0	47.9	1.9
15	27.7	70.9	2.6
			Mean: 2.2

The radioiodine concentrations of the colloid may, therefore, be used to calculate the rates of accumulation and discharge of iodine. In calculating the rate of accumulation from equation 4, it was necessary to assume that the specific activity of the iodide in the extra-cellular fluid surrounding all follicles in the gland was identical. This was felt to be a reasonable assumption since the iodide ion may be expected to diffuse with sufficient rapidity to insure a uniform extra-cellular specific activity.

On the other hand, the calculation of the rate of discharge of organic iodine from the follicle on the basis of equation 7, required the assumption that the concentration of dischargeable colloid iodine be identical from follicle to follicle. However, Gersh and Caspersson (1940) in U-V absorption studies of single follicle sections, demonstrated that there is a marked variation in the organic iodine content from follicle to follicle. But this variation could be reduced if the animals were given supplementary iodide feedings. Purina Fox Chow, the rat food used in the present experiments, was known to possess a relatively high iodine content (the amount ingested per day supplying about 10 times the daily need of iodine), and, therefore, may have fulfilled the requirements for colloid homogeneity.

Another difficulty encountered in the measurements of iodine discharge was that, while the determination of the rate of iodide entry was based on the analysis of a series of follicle sections from the same gland, that of

iodine discharge required a comparison of results obtained in different animals, and thus the possibility of heterogeneity was further increased.

Accordingly, it was felt that the determination of the rate of accumulation of iodide was more accurate than that of the rate of discharge of iodine.

Rate of iodine metabolism as a function of the surface area of the follicle

At the early intervals after injection of radioiodine, a mere examination of radioautographs suggested a faster pick-up of the element by small than by large follicles (Figs. 4–6). A definite proof that this was actually the case was obtained by quantitative radioautography (Fig. 2).

At the late intervals after injection of radioiodine, a mere examination of radioautographic images indicated that the radioiodine had been released by the small and not by the large follicles (Figs. 8 and 9). Quantitative data proved that the rate of iodine discharge was indeed faster in small than in large follicles (Figs. 2 and 3).

The faster turnover of the small follicles (Table 2) might be due to the fact that their surface area per unit volume is greater than in large follicles. This possibility was examined by calculating the rates of iodine accumulation and discharge per unit surface area of colloid. The rate of iodine accumulation expressed in this manner was found to be approximately the same in all but very small and very large follicles (Fig. 10). Similarly, the rate of iodine discharge per unit surface area of colloid was the same for all follicles of intermediate size (Fig. 10).

If the rate of iodine metabolism is directly related to the surface area of the colloid, it follows that this rate will be enhanced by mechanisms increasing the ratio of surface to volume. This may be produced by the splitting of a large follicle into two or more smaller follicles—a process taking place in the normal development of the thyroid gland (Leblond and Peets, 1951; Leblond, Peets and Nadler, unpublished)—or by transformation of the follicle into a tubular or other non-spherical structure—a process known to occur in the general hypertrophy of the gland seen during thiouracil treatment or after feeding a diet low in iodine.

The conclusion that the functional activity of the follicle is directly related to the surface of the colloid is strictly valid only for middle-sized follicles, since the extremes of follicle size showed opposite trends for the rate of iodine accumulation and discharge (Fig. 10). Further interpretation of these results was attempted using the data on accumulation rate which, as stated above, are more valid than those on discharge rate.

Since the extra-cellular iodide came in contact with the outer follicle surface rather than the colloid surface, it was felt that the linearity of the graph depicted in Figure 10 might be improved by referring the rates of iodide accumulation to the outer surface of the follicle rather than to that

of the colloid. To recalculate rates per unit surface area of follicle, it was first necessary to take measurements of the mean epithelial height. This height was found to be statistically the same in all follicle classes (Table 4). The rates of iodide accumulation per unit follicle surface area (Table 4, column 2) were found to differ from the mean (100) by about as much as those obtained per unit colloid surface area (column 1), but in an opposite manner. However, when calculations were based on a surface area intermediate between that of the colloid and follicle itself, figures close to the mean (100) were obtained, as shown in Table 4, column 3. (This area, referred to as the "effective follicle surface," is characterized by a radius equal to that of the colloid plus 1/2 of the epithelial height.) Therefore,

TABLE 4. RATE OF ACCUMULATION OF IODIDE EXPRESSED PER UNIT AREA OF (1) COLLOID, (2) FOLLICLE AND (3) EFFECTIVE* FOLLICLE SURFACE IN THYROID FOLLICLES OF VARIOUS SIZES

Colloid diameter (microns)	Mean epithelial height (±std. error) (microns)	Accumulation rate		
		(1) Per unit colloid surface area	(2) Per unit follicle surface area	(3) Per unit effective* follicle surface area
		(Arbitrary units: % of mean)		
90	7.2±0.53	68	113	101
70	7.0±0.57	93	120	106
50	7.1±0.62	91	104	98
40	7.2±0.38	93	96	95
30	7.1±0.18	104	90	96
25	6.9±0.18	115	88	101
20	6.9±0.19	132	85	101

* Effective follicle radius = colloid radius + ½ epithelial height.

the rate of accumulation of iodide, calculated per unit of this effective follicle surface area, was the same for follicles of all sizes.

The proportionality between the rate of iodide accumulation and the effective surface area is of interest in view of the finding that in young adult rats similar to those used here the *number* of epithelial cells in any follicle was proportional to the area of the effective follicle surface (Leblond, Peets and Nadler, unpublished). Thus, the rate of iodide accumulation was also proportional to the number of cells present in the follicle. Therefore, the rate of iodide accumulation by every thyroid cell in every follicle must be identical.

The control of iodide accumulation by the effective follicle surface might be explained by assuming that, although the entry of iodide takes place over the entire outer surface, the rate of its accumulation is governed by an intracellular structure such as the Golgi zone or, more likely, by the volume of the whole cell. For, it can be shown that the effective follicle surface is almost exactly proportional to the *total cell volume* of the follicle (Appendix

4); and, therefore, the rate of iodide accumulation is also almost exactly proportional to the total cell volume of any given follicle. This conclusion is in agreement with previous results indicating that the uptake of iodide by the whole gland depends on the total epithelial cell volume in normal and hypophysectomized rats (Leblond and Süe, 1941). The total epithelial cell volume thus appears to be the ultimate factor controlling iodide accumulation in the thyroid follicle, as well as in the whole gland, of normal young adult rats.

Assuming the existence of a steady state with respect to the quantity of iodine in individual follicles, the rate of iodine discharge should have the same dependence on follicle surface area as the rate of iodide accumulation —(a fact in agreement with the data in Figure 10 in the case of follicles of intermediate size). Therefore, it appears likely that all activities of the follicle depend on its effective surface area and, thus, on the total volume of the cells which form its epithelium.

SUMMARY

The rates of accumulation (i.e., organic binding) and discharge of iodine in thyroid follicles of various sizes were investigated in male rats of 100–125 gm. sacrificed at various time intervals after radioiodine administration. Radioautographs of the thyroids were prepared and the intensity of the radioautographic images was determined by counting the number of silver grains present in the photographic emulsion. By analysis of these data, the relative concentrations of radioiodine, as well as the rates of iodine accumulation and discharge, were calculated for follicles of various sizes.

The small follicles—generally located in the center of the gland—accumulate radioiodine in their colloid more actively, achieve a greater concentration of this element, and discharge it at a faster rate than large follicles. Accordingly, the biological half-life of the iodine present in the follicle colloid increases with the size of the follicle.

An analysis of these findings indicates that the rate of iodide accumulation by any thyroid follicle is proportional to its "effective follicle surface," the area of which is slightly smaller than that of the outer surface of the follicle. This area is also proportional to the number of cells in the follicle. Such a conclusion implies that every cell in every follicle is accumulating iodide at the same rate.

The significance of the effective follicle surface depends upon the fact that this surface is very nearly proportional to the total volume of the epithelial cells in the follicle. The ultimate factor controlling iodide accumulation, and presumably iodine metabolism as well seems to be the total epithelial cell volume. Thus, for the whole gland, the rate of iodine metabolism depends on the total cell volume of the aggregate of follicles which together make up the thyroid gland.

APPENDIX I

Calculation of the radioiodine concentration as a function of the true colloid diameter of the thyroid follicle

Suppose that the thyroid gland is made up of q sets of spherical follicles of which the true number in each set is N_1, N_2, N_3, $\cdots N_q$, and the true mean colloid diameter, D_1, D_2, $D_3 \cdots D_q$, respectively ($D_1 > D_2 > \cdots D_q$).

Referring to Fig. 13, consider any sphere of which, for the sake of illustration, the diameter is 50 (in arbitrary units). Let the plane of the figure be perpendicular to the cutting plane of the histological sectioning apparatus. Take specific planes parallel to the histological section such that the diameters are 40, 30, and 20 (Fig. 13 is drawn to scale). Let S_{40}, S_{30} and S_{20} be the respective distances of these planes from the diametrical

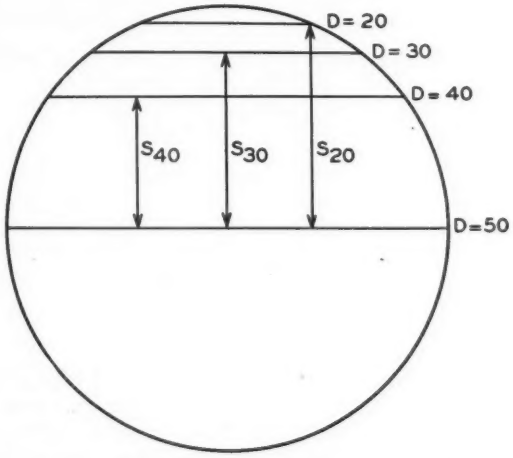


FIG. 13. Representation of a sphere (diameter D; radius R) to examine the probability of slicing this sphere into random sections with diameters within given limits. Assuming $D=50$ arbitrary units, the probability of slicing this sphere into random sections with diameters between 40 and 50 is designated by P_{40-50} and can be calculated by the ratio S_{40}/R where S_{40} is the distance between the two chords $D=50$ and $D=40$. The remaining P values; e.g. P_{30-40}, P_{20-30}, etc., can be determined in a similar manner.

plane of the sphere. These values are easily calculated since, here, $S_{40} = \sqrt{(25)^2-(20)^2}$; $S_{30} = \sqrt{(25)^2-(15)^2}$; etc. Then, the probability, P_{40-50}, of sectioning through the sphere a circular plane whose diameter lies between 40–50 is $(S_{40})/R$, where R is the radius. Similarly P_{30-40} is $(S_{30}-S_{40})/R$ and $P_{20-30}=(S_{20}-S_{30})/R$, etc. Accordingly, by this method, for a sphere of given true diameter, D, the probability, ${}_DP_{m-n}$, of its appearance in section between any two diameters d_m and d_n ($d_m < d_n < D$) can be calculated (Table 5).

Thus, for the above postulated thyroid sets of follicles, the probable number of contributions, for example, from the sets whose diameters are greater than d_n to sections with diameters between d_m and d_n is: $N_{1\,D_1} P_{m-n} + N_{2\,D_2} P_{m-n} + N_{3\,D_3} P_{m-n} + \cdots$, to include all sets of diameters greater than or equal to d_m.

Suppose, now, that the mean radioiodine concentration of each follicle set for any given tissue is c_1, c_2, c_3, $\cdots cq$. Then, for the above illustration, the average radioiodine

TABLE 5. PER CENT PROBABILITY OF A GIVEN SPHERE BEING SECTIONED INTO CIRCULAR DISCS WHOSE DIAMETERS ARE WITHIN A CERTAIN RANGE

(Arbitrary Units)

Diameter of sphere	Diameter range of section								
	10–15	15–20	20–25	25–30	30–35	35–40	40–50	50–70	70–90
90	0.7	1.1	1.3	1.8	2.4	2.4	6.7	20.4	62.6
70	1.2	2.3	2.6	3.1	3.4	4.6	12.0	70.3	
50	2.8	3.6	5.2	6.4	8.4	11.6	60.0		
40	4.0	6.5	8.5	12.0	17.5	48.5			
35	5.7	7.5	10.3	18.9	51.5				
30	8.0	11.3	19.3	55.2					
25	11.2	20.0	60.0						
20	21.0	66.0							
15	74.5								

concentration, $\bar{c}_{m-n}$, of the follicle sections with colloid section diameters between d_m and d_n is:

$$\frac{N_{1\,D_1} P_{m-n}\, c_1 + N_{2\,D_2} P_{m-n}\, c_2 + \cdots}{N_{1\,D_1} P_{m-n} + N_{2\,D_2} P_{m-n} + \cdots} \tag{A1}$$

This method, thus, can be used to determine the colloid radioiodine concentration, c, as a function of the true colloid diameter, D, from a knowledge of the mean value of c for the section diameters (crude diameters).

Specifically, the procedure is as follows: subdivide the follicles counted into sets whose true colloid diameters are 90, 70, 50, 40, 35, 30, 25, 20, and 15 microns, and let the numbers of follicles in each set be N_{90}, N_{70}, N_{50}, N_{40}, N_{35}, N_{30}, N_{25}, N_{20} and N_{15}, respectively. Calculate and tabulate the P values: ${}_{90}P_{70-90}$, ${}_{90}P_{50-70}$, ${}_{90}P_{40-50}$ $\cdots$; ${}_{70}P_{50-70}$, ${}_{70}P_{40-50}$ $\cdots$; etc. (Table 5).

Let the counted number of follicles with colloid section diameters 70-90 μ, 50-70 μ, etc., be U_{70-90}, U_{50-70}, U_{40-50}, etc., respectively. Suppose, as before, that the mean radioiodine concentration of a follicle with true colloid diameter, D, is c_D, and of the aggregate of sectioned follicles with crude diameter between d_m and d_n is $\bar{c}_{m-n}$.

Hence, starting with the largest follicles,

$$U_{70-90} = N_{90\ 90} P_{70-90},$$

whence, determine N_{90}. Then, $N_{90\ 90}P_{m-n}$ can be calculated for any m and n. Again,

$$U_{50-70} = N_{90\ 90} P_{50-70} + N_{70\ 70} P_{50-70},$$

whence, determine N_{70} and calculate $N_{70\ 70}P_{m-n}$. As before,

$$U_{40-50} = N_{90\ 90} P_{40-50} + N_{70\ 70} P_{40-50} + N_{50\ 50} P_{40-50},$$

whence, determine N_{50} and so forth, for N_{40}, N_{35}, N_{30}, N_{25}, N_{20}, and N_{15}.

Finally, from equation A1,

$$\bar{c}_{70-90} = \frac{N_{90\ 90} P_{70-90}\, c_{90}}{N_{90\ 90} P_{70-90}},$$

whence, determine c_{90} which is equal to $\bar{c}_{70-90}$. Again,

$$\bar{c}_{50-70} = \frac{N_{90\ 90} P_{50-70}\, c_{90} + N_{70\ 70} P_{50-70}\, c_{70}}{N_{90\ 90} P_{50-70} + N_{70\ 70} P_{50-70}},$$

so that c_{70} can be calculated since $\bar{c}_{50-70}$ is known experimentally, ${}_D P_{m-n}$ is calculated as above (Table 5), and N_D is determined as explained above. By tabulating these results,

the procedure can be systematically applied to obtain the remaining values c_{50}, c_{40}, c_{35}, c_{30}, c_{25}, c_{20}, and c_{15}.

Therefore, by the foregoing analytic technique, the relative radioiodine concentration per true colloid diameter can be deduced from the results obtained per crude diameter.

APPENDIX 2

Calculation of the biological half-life of the colloid organic iodine in the thyroid follicle

Let t' be the half-life of the colloid organic iodine. Then, from equation 7, for any two late time intervals after the administration of radioiodine, t_1 and t_2:

$$t_1 - t_2 = -\frac{DM}{q_0} \ln \frac{c_1}{c_2}. \tag{A2}$$

The quantity $-(q_0/DM)$ can be determined from the slope, l, of the plot $ln\ c$ vs. t. By definition t' is a time interval such that $c_1/c_2 = 2$. To calculate t', put $t' = t_2 - t_1$. Hence, from equation A2,

$$t' = -\frac{0.693}{l}. \tag{A3}$$

APPENDIX 3

Relation between exposure time (T) and the amount of radiation received by the photographic emulsion (radiation exposure, E_T)

From the fundamental law of radioactivity, the intensity of radiation, I_t, at any time, t, is:

$$I_t = I_0 e^{-\lambda dt},$$

where λ is the disintegration constant of the isotope. By definition, the photographic radiation exposure over a period of time, T (exposure time), is:

$$E_T = \int_0^T I dt = \frac{I_0}{\lambda}(1 - e^{-\lambda T}). \tag{A4}$$

Thus, for a given radiation intensity, the relative exposures may be calculated for various values of T.

APPENDIX 4

Relation between the rate of iodide accumulation per unit "effective follicle surface" area and that per unit cell volume

Let R be the outer follicle radius, r be the inner follicle radius (that is, of the colloid), then, h, the epithelial height, is equal to $R-r$, and, $r+(h/2)$ is the "effective follicle radius."

The total cell volume of the follicle is $4/3\pi(R^3-r^3)$ which, by replacing R by $h+r$, reduces to $4\pi h[r^2+hr+(h^2/3)]$.

The "effective follicle surface" area is $4\pi[r+(h/2)]^2$ which is equivalent to $4\pi[r^2+hr+(h^2/4)]$.

It is evident that for $h=7\mu$ and $r>10\mu$ (that is, for any values of h or r likely to be encountered in a thyroid follicle), the ratio of cell volume to "effective follicle surface" area is almost exactly equal to the epithelial height, h. Since h is constant in all types of follicles in the animals used (Table 4), the ratio is likewise constant. Hence, since the rate of accumulation of iodine per unit "effective follicle surface" area is constant, it is also constant per unit cell volume.

Acknowledgments

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FALL IN BLOOD SUGAR FROM ANTERIOR PITUITARY EXTRACT[1]

V. W. WESTERMEYER[2] AND M. S. RABEN[3]

The Ziskind Research Laboratories, New England Center Hospital, and the Department of Medicine, Tufts College Medical School, Boston, Massachusetts

THE fall in blood sugar produced by anterior pituitary extracts has been found with preparations of widely different preparative histories and containing different associated pituitary hormones. Thus, Horsters (1933) employed the thyrotropin preparation of Junkmann and Schoeller (1932) to lower the blood sugar of rabbits and certain human patients, while Hoffmann and Anselmino (1933) obtained this effect in dogs with a thyrotropin-free ultrafiltrate of pituitary extract. Collip and co-workers observed small changes in several species with an alcoholic pituitary extract and with a preparation which had been boiled for 15 minutes at pH 10 (Collip, 1940; O'Donovan and Collip, 1938). More recently, Millman and Russell (1950) found the crystalline growth hormone preparation of Wilhelmi, Fishman and Russell (1948) effective in lowering the blood sugar of rats, and attributed the hypoglycemic effect to growth hormone. This preparation was also shown to have anti-insulin properties in the same species.

The present paper is a report of studies with a pig anterior lobe pituitary preparation purified by the glacial acetic acid-oxycellulose method of Astwood, Raben, Payne, and Grady (1951). This fraction is known to be rich in corticotropin and intermedin, and has been shown by Rosenberg (1953 a and b) to be potent in adipokinin, to increase oxygen consumption, and to inhibit the rise in respiratory quotient after administered glucose. It contains no more than traces of the other known pituitary hormones (Raben, Rosenberg, and Astwood, 1952). Studies with this preparation show that it is highly potent in lowering the blood sugar concentration of mice, and that it appears to have an anti-insulin effect not attributable to its corticotropin content.

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[2] At present, Research Fellow, The Arthritis and Rheumatism Foundation. This work was done largely during tenure of a Clinical Fellowship from the New England Chapter, The Arthritis and Rheumatism Foundation.

[3] Lowell M. Palmer Senior Fellow.

METHODS

Female mice of the Swiss strain were obtained from Taconic Farms weighing 18 to 20 grams and were usually used within a two-week period. The animals were fed Purina Fox Checkers and unless otherwise stated, were not fasted before the beginning of each experiment, but had no food or water available during the experiment. Blood sugar was determined by the method of Nelson (1944) adapted to allow duplicate determinations on 0.1 ml. blood obtained without anesthesia or anti-coagulant from the tail. Before bleeding, the animals were warmed under an infrared lamp for one to two minutes. The mice were bled twice, just prior to injection with the test substance, and again at the stated interval. Adrenalectomy was performed under ether anesthesia, through a mid-line lumbodorsal incision. The oxycel-purified pituitary extract is the "oxycel-purified corticotropin" or "Preparation D" of previous communications from this laboratory. This material, stored in the refrigerator in solution in 0.1 N HCl, was alkalinized and diluted just prior to use. The growth hormone preparations employed were stored as the dry powder and dissolved just before use. All pituitary preparations were brought to approximately pH 8.5 with NaOH and injected intraperitoneally in a volume of 0.2 cc. Insulin was a commercial Lilly crystalline zinc preparation.

RESULTS

The effect of the oxycel-purified pituitary extract and the growth hormone preparations on the blood sugar concentration of unfasted intact mice is summarized in Table 1. A lowering of the blood sugar concentration was apparent at 30 but not at 15 minutes after injection of the oxycel-purified preparation. It may be seen that the oxycel preparation was considerably more potent than the growth hormone preparations. With all preparations, a greater depression of blood sugar was obtained with the same dose in a 3-hour period after injection than in a 1-hour period, but with the larger doses of the oxycel preparation a near-maximum fall was produced in 1 hour. Thus, 100 μg. of the oxycel preparation produced a 55% fall in blood sugar in 1 hour, and only a slightly larger fall (60%) in 3 hours. A dose of 1 μg. of the oxycel fraction, however, produced no fall in 1 hour but did lower the sugar 17% in 3 hours. A dose of 100 μg. appeared to cause the maximal fall obtainable in the 1-hour period, a dose of 1 mg. causing no greater fall, and, indeed, a somewhat smaller response was elicited with the 1 mg. dose, Uninjected animals and injected controls had little change in blood sugar in either the 1-hour or 3-hour period.

An exact estimate of the relative potencies of the preparations tested is probably not possible from the data in Table 1. It would appear, however, that the oxycel fraction was many times as potent as the Armour growth hormone preparation. A greater response was obtained with 100 μg. of the oxycel fraction than with 5 mg. of the Armour growth hormone in both the 1-hour and the 3-hour test. The Armour growth hormone, in turn, seemed to be more potent than the glacial acetic acid extracted growth hormone preparation (Raben and Westermeyer, 1951) in producing blood sugar depression.

TABLE 1. EFFECT OF PITUITARY PREPARATIONS ON THE BLOOD SUGAR CONCENTRATION OF INTACT MICE

Preparation	Dose	Number of determinations	Test period, hours	Average initial blood sugar, mg. %	Average final blood sugar, mg. %	Mean change in blood sugar, %
Control		4	¼	144	170	+13
Control		5	½	118	141	+18
Control		26	1	135	134	− 1
Control[1]		5	1	123	117	− 4
Control		24	3	141	134	− 1
Control[2]		8	3	177	179	+ 1
Oxycel preparation	100 μg.	3	¼	134	131	− 1
Oxycel preparation	100 μg.	2	½	100	79	−21
Oxycel preparation	1 μg.	5	1	109	119	+11
Oxycel preparation	3 μg.	19	1	151	126	−18
Oxycel preparation	10 μg.	28	1	138	92	−28
Oxycel preparation	30 μg.	16	1	131	89	−32
Oxycel preparation	100 μg.	8	1	168	76	−55
Oxycel preparation	1 mg.	10	1	136	75	−44
Oxycel preparation	0.1 μg.	16	3	166	156	− 6
Oxycel preparation	1 μg.	8	3	138	115	−17
Oxycel preparation	100 μg.	4	3	167	66	−60
Armour growth hormone[3]	100 μg.	4	1	154	144	− 8
Armour growth hormone	1 mg.	10	1	138	137	− 1
Armour growth hormone	5 mg.	8	1	162	113	−30
Armour growth hormone	1 μg.	8	3	145	126	−13
Armour growth hormone	10 μg.	8	3	152	124	−18
Armour growth hormone	100 μg.	8	3	152	117	−24
Armour growth hormone	1 mg.	8	3	123	102	−16
Armour growth hormone	5 mg.	8	3	153	90	−40
Glacial acetic extracted growth hormone	5 mg.	8	1	154	156	+ 2
Glacial acetic extracted growth hormone	100 μg.	8	3	164	154	− 5
Glacial acetic extracted growth hormone	1 mg.	14	3	138	125	−00
Glacial acetic extracted growth hormone	5 mg.	7	3	132	101	−24

0.1 c.c. of blood was taken from unfasted female mice for initial blood sugar values, the animals were injected intraperitoneally with the pituitary preparation in 0.2 cc. mildly alkaline solution and bled again at the stated interval. Food and water was not available during the experiment. The control mice except as specified, received no injections.

[1] 2 cc. normal saline.

[2] 0.2 cc. water at pH 9 with NaOH.

[3] Lot #R285128.

The 1-hour dose-response relationship of the oxycel-purified preparation is plotted in Figure 1 with the uninjected control values added in the left lower corner. It can be seen that under the conditions of the experiment a reasonable log dose-response relation exists for doses between 3 μg. and 100 μg.

Adrenalectomized animals, with and without cortisone maintenance, were used to determine whether adrenal steroids modified the blood sugar response. From Figure 2, it appears that neither adrenalectomy nor the

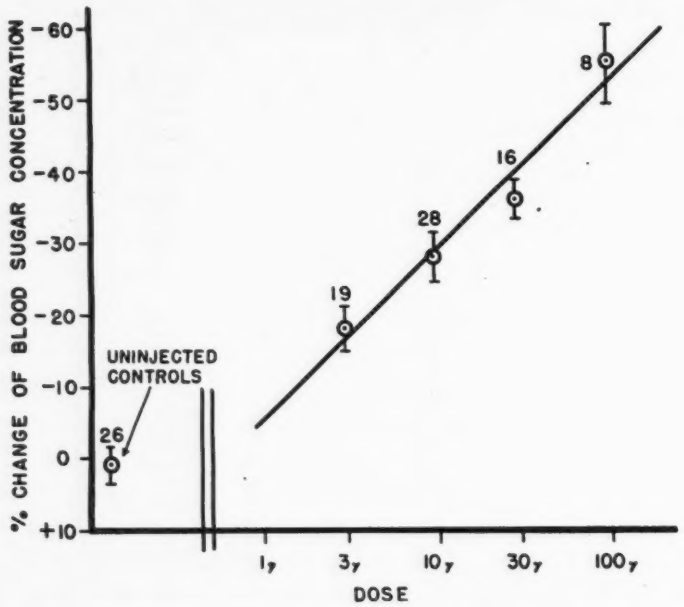

FIG. 1. Log dose-response curve of the 1 hour effect of the oxycel-purified pituitary extract on the blood sugar concentration of intact unfasted mice. Each point is the mean of the number of determinations indicated. The ordinate shows the per cent change in blood sugar; the uninjected control value is shown in the left lower corner. The bars extending from each point represent the standard errors of the means.

type of supportive therapy greatly modifies the blood sugar-depressing effect of this preparation.

Early in this study it was noticed that, although there was a marked reduction in blood sugar in one hour after injection of the larger doses of oxycel fraction, abnormally low levels were only occasionally reached at any interval and convulsions did not occur. To determine whether this was due merely to the brevity of action of the pituitary fraction or whether it represented the maximal effect obtainable, the effect of a second injection was determined. Four intact and five cortisone-treated adrenalectomized mice were injected with 100 μg. of the oxycel-purified material. After one hour, each animal was bled and the injection repeated. No fall in blood sugar values occurred in the one-hour period after the second injection.

In another attempt to modify the blood sugar-depressing effect, 16 mice were divided into two groups, one of which received seven daily injections of 100 μg. of the oxycel-purified material while the other received a similar course of 1 mg. of Armour growth hormone. On the eighth

day the unfasted animals were bled for blood sugar determination, the first group was immediately given 100 μg. of the oxycel-purified material and the second, 5 mg. of Armour growth hormone. The blood sugar fall in these animals was similar to that previously found in animals not pretreated. The oxycel fraction caused a fall of 41% in 1 hour (4 mice), and 38% in 3 hours (4 mice) and the growth hormone 24% in 1 hour (3 mice) and 45% in 3 hours (4 mice).

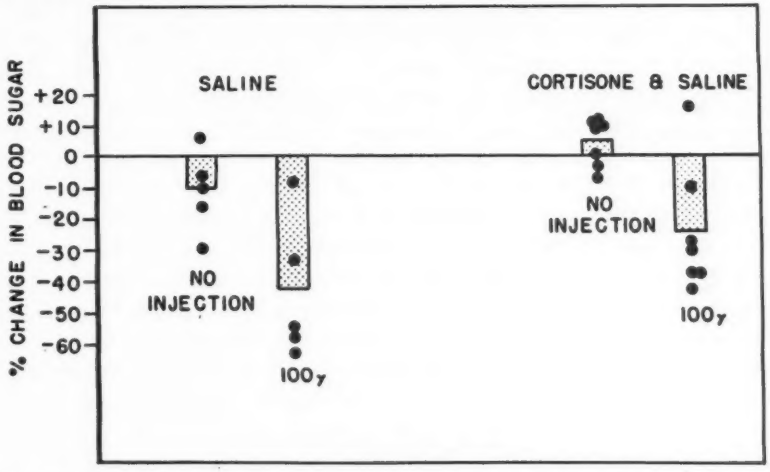

FIG. 2. Fall in blood sugar in adrenalectomized mice. The change in blood sugar at 1 hour after injection with 100 μg. of the oxycel-purified material is compared with uninjected controls using unfasted mice adrenalectomized 3 days earlier. The left pair of graphs summarizes an experiment using mice given 1% NaCl in the drinking water; the results on the right were obtained with animals receiving 2.5 mg. cortisone subcutaneously immediately after operation and saline as well.

The oxycel preparation was tested in mice fasted for 18 hours; 100 μg. produced an average fall in blood sugar of 27% in one hour (4 mice). This fall was less than would be expected in unfasted animals, but too few mice were used to establish this point.

The oxycel-purified extract was found to protect mice against insulin convulsions and to inhibit the hypoglycemic effect of insulin. The effect was studied in adrenalectomized mice to exclude similar effects from the corticotropic activity of the preparation. Table 2 indicates the protection of fasted, cortisone-treated adrenalectomized mice against insulin-induced convulsions. In the experiment summarized in Table 3, the cortisone-treated adrenalectomized mice were not fasted before the experiment, but were fasted during the 6½-hour observation period. The subcutaneous injection of 5 units of insulin produced a fall in blood sugar of 74.3 per cent

TABLE 2. PROTECTION AGAINST INSULIN-INDUCED CONVULSIONS IN ADRENALECTOMIZED MICE

Experiment	No mice	Insulin units	Oxycel preparation, micrograms	Convulsions within 1 hour, %	Convulsions within 2 hours, %
I	5	0.5	100	0	40
	5	0.5	0	80	100
	5	1.0	100	0	40
	5	1.0	0	100	100
II	5	1.0	25	40	80
	5	1.0	10	20	40
	5	1.0	0	80	100

2.5 mg. of cortisone acetate was injected subcutaneously into adrenalectomized mice 4 days before the experiment; 1% NaCl solution was used as drinking fluid. Subcutaneous insulin, or intraperitoneal pituitary extract followed immediately by insulin was administered after 18–22 hours of fasting. Room temperature was 24° C.

in 90 minutes and convulsions during the 6½-hour period in four of the six unprotected animals, whereas those animals receiving 100 μg. of the pituitary preparation in addition to insulin had a 90-minute fall in blood sugar of 55.3% and convulsions in none of the six animals. It is of interest that the blood sugar fall with 100 μg. of the pituitary extract plus 5 units of insulin was similar to the fall expected from 100 μg. of the pituitary preparation alone.

DISCUSSION

Horsters (1933) considered the pituitary hypoglycemic effect to be an insulin-like action, but Hoffman and Anselmino (1933) attributed it to a pancreotropic effect producing increased insulin secretion through stimulation of the Islets of Langerhans. In support of the latter view was the failure of pancreatectomized dogs to respond as did normal dogs with a fall of blood sugar. Several findings in the present experiments seem difficult to explain on the assumption that the depression of blood sugar

TABLE 3. INHIBITION OF INSULIN-INDUCED HYPOGLYCEMIA IN ADRENALECTOMIZED MICE

No. mice	Insulin units	Oxycel preparation, micrograms	Avr. initial blood sugar mg. %	Avr. blood sugar at 90 minutes, mg. %	Mean decrease in blood sugar in 90 minutes, %	Convulsions within 6½ hrs. % of mice
6	5	100	122	54.5	55.3	0
6	5	0	122	30.2	74.3	67

Mice received 1.25 mg. cortisone acetate subcutaneously at the time of adrenalectomy and were allowed to remain in cages with food and 1% NaCl solution available until beginning of experiment 2 days later. Insulin was injected subcutaneously and the pituitary preparation, when administered, was injected intraperitoneally immediately before the insulin. Room temperature was 24° C. The times of onset of convulsions were 1½, 2¼ 3¼ and 4½ hours after the insulin injection.

results from increased insulin secretion. Fasted animals were no more, and perhaps less, susceptible to the hypoglycemic effect than were non-fasted animals; despite the rapid rate of fall in blood sugar concentration that occurred in the first hour after injection of a maximal dose (100 μg.), little or no further decline was observed and very low values were not reached; the administration of a second dose of 100 μg. one hour after the first produced no further blood sugar change; the preparation used appeared to inhibit the hypoglycemic effect of administered insulin.

It is of interest that in the studies of Millman and Russell (1950) both hypoglycemic and anti-insulin effects were obtained in rats with a crystalline growth hormone preparation. However, no correlation between growth hormone activity and the blood sugar-depressing effect can be shown in the present experiments. The oxycel-purified material is not growth-promoting by a standard assay in hypophysectomized rats, yet it is considerably more potent in producing blood sugar depression in mice than the growth hormone preparations studied.

The oxycel fraction has also been shown to be more potent in adipokinetic activity than purified growth hormone preparations (Rosenberg, 1953a). Adrenal steroid is necessary for fat mobilization (Payne, 1949; Rosenberg, 1953a) but is not required for the blood sugar effect. Thereis, however, little basis on which to decide at present whether or not one substance accounts for the lowered blood sugar and the fat mobilization, as well as for the anti-insulin, and the other metabolic effects of the oyxcel fraction.

SUMMARY

An anterior pituitary preparation, purified by the glacial acetic acid-oxycellulose method, lowered the blood sugar concentration in normal and adrenalectomized female mice, and inhibited the effect of insulin in the adrenalectomized animal.

The preparation which produced these effects is rich in corticotropin and intermedin and it exerts other metabolic effects, but contains no more than traces of the other well-defined pituitary hormones.

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EVALUATION OF THE ANTITHYROID ACTIVITY OF 5-IODO-2-THIOURACIL[1]

R. C. GOLDBERG AND J. WOLFF[2]

Biological Laboratories, Harvard School of Dental Medicine and Harvard Medical School, Boston, Massachusetts

INTRODUCTION

AMONG the known types of antithyroid agents, such as the thiocarbamides, anions such as thiocyanate or perchlorate, anilines and polyphenols, and iodide, only the last named fails to exhibit goitrogenic action. Recently it has been claimed by several investigators that 5-iodo-2-thiouracil (ITU) is a potent antithyroid drug and yet has little or no goitrogenic activity. Thus, Gassner *et al.* (1950) reported that while ITU effected a significant reduction in the oxygen consumption of rats and inhibited the thyroidal uptake of radioactive iodine, the thyroid glands from these animals were but slightly hypertrophied and revealed little or no histological evidence of enhanced activity. Equimolar inorganic iodide administered with thiouracil (TU) failed to duplicate this apparently anomalous action of ITU. Furthermore, it was suggested that, perhaps, ITU *per se* inhibited thyrotrophic hormone (TSH). Studies by Barrett and Gassner (1951) and Barrett *et al.* (1951) indicated that while ITU effected about the same lowering of oxygen consumption as did equimolar quantities of TU (or TU and iodide), it had little effect on thyroid weight, effected no essential histological change in the thyroid gland, and resulted in a decreased loss of thyroid iodine (as compared to TU or TU plus iodide). More recently Albert and Ford (1952) found that the antithyroid activity of ITU, as measured by its effect on the rate of loss of thyroidal I^{131}, was as great as that of TU.

It thus seemed of interest to investigate in some detail the question as to whether ITU does actually block hormone synthesis in the thyroid without goiter formation or whether this "unique property" could equally well be explained by hydrolysis of the drug and the combined action of the products.

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[2] Present address: Massachusetts General Hospital, Boston, Mass.

EXPERIMENTAL

Sprague-Dawley rats, maintained on Purina Lab Chow or on the Remington (1937) low iodine diet (#342 with 2% desiccated liver) were used in all experiments. The antithyroid agents[3] and iodide (as NaI), used in this study, were thoroughly mixed with finely ground chow. The iodide administered was always equimolar to ITU. QO_2 studies on liver and kidney (approximately 40 mg. tissue wet weight per bath, sliced free hand) were carried out in Krebs-Hensleit (1932) phosphate buffer at pH 7.4 in an O_2 atmosphere in the conventional Warburg apparatus. Incubation was carried out for two hours.

In studies on uptake of P^{32} by thyroid tissue, approximately 45 microcuries of P^{32} were injected intraperitoneally and the rats were sacrificed 1 or $1\frac{1}{2}$ hours later. The thyroid glands were removed, weighed, digested in 1 ml. 2 N NaOH, and counted with an end window G-M tube.

Release of thyroidal I^{131} was measured by external gamma counting as described by Wolff (1951).

For the determination of thyroid gland iodine (I^{127}) the thyroid glands were homogenized in 1 ml. of cold 10% trichloracetic acid (TCA) in an all glass homogenizer, the TCA precipitates were washed twice with 2.5 ml. cold 5% TCA, the precipitates and combined supernatants were then analyzed for iodine by the method described by Taurog and Chaikoff (1946).

For histological examination, the thyroid glands were fixed in formalin and routinely stained with hematoxylin and eosin. Piutitary glands were prepared for cytological examination by methods described elsewhere (Goldberg and Chaikoff, 1952).

I. *The effect of ITU, propylthiouracil (PTU), and PTU plus I^- on the respiration of kidney and liver tissue, thyroid gland weight and histology, pituitary cytology, and growth*

It will be seen from Table 1 that the administration of 0.16 or 0.2% ITU (in the diet) to male Sprague-Dawley rats for a period of seven weeks resulted in a slight reduction in the respiration of surviving liver slices and questionable depression of kidney slice respiration.

In agreement with the results of Barrett and coworkers (1951) and Gassner *et al.* (1950), ITU administration at a level of 0.2% resulted in a relatively slight increase in the size of the thyroid gland (Table 1) as compared to the increase noted with PTU. The addition of an equivalent amount of

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[3] We are grateful to Dr. Stanton Hardy of Lederle Laboratories, Pearl River, N. Y., for his gift of thiouracil and to Dr. E. Oppenheimer of CIBA Pharmaceutical Products, Inc., Summit, N. J., for supplying the iodothiouracil used in this study.

TABLE 1. THE EFFECT OF ITU AND PTU ON LIVER AND KIDNEY QO_2 AND ON THE STRUCTURE OF THE THYROID AND PITUITARY GLANDS

Expt.	Gp.	Treatment	Initial body wt. (mean)	Final body wt. (mean)	QO_2 kidney		Thyroid gland wt.	Histology	
								Thyroid gland	Pituitary gland
			gm.	gm.	$\mu L\ O_2/mg./hr.$	"P"	mg./100 gm.		
I	A	0.16% ITU (7)*	119	245	17.8±0.8†	<.05	8.8±1.9†	Normal	Beta cells: hypertrophy, hyperplasia, degranulation Alpha cells: incomplete degranulation
	B	0.1% PTU (7)	121	172	15.9±1.2	<.01	36.6±6.8	H. and H.‡	Beta cells: hypertrophy, hyperplasia, degranulation Alpha cells: total degranulation
	C	0.1% PTU plus I^- (7)	122	178	—	—	24.7±6.2	As B	Same as B
	D	I^- (7)	120	287	—	—	6.6±0.5	Normal	Normal
	E	Control (7)	121	287	19.1±1.1	—	5.7±0.6	Normal	Normal
					QO_2 liver				
II	F	0.2% ITU (5)	205	215	5.6±0.3	<.05	13.0±1.8	Normal	Same as A
	G	0.1% PTU (5)	210	210	4.9±0.4	<.01	33.0±3.0	H. and H.	Same as B
	H	Control (5)	200	215	6.5±0.4	—	8.5±1.8	Normal	Normal

Expt. I: Male Sprague-Dawley rats maintained for seven weeks on augmented diets.
Expt. II: Female Sprague-Dawley rats maintained for four weeks on augmented diets.
* Number of rats/group.
† Mean ± Standard Deviation.
‡ Hypertrophy, hyperplasia, colloid loss, high vascularity.
"P" (Fisher "t-test") value relating to control slices

iodide to PTU, limited the increase in gland weight only by approximately one-third. Similarly, little or no histological differences could be noted between control thyroid glands and those from ITU-treated rats in contrast to the picture of intense cellular hypertrophy and hyperplasia, colloid loss, high vascularity, etc., presented by the thyroids of the PTU (or PTU plus I^-) treated rats.

Cytological examination of the pituitary glands from rats treated with PTU, PTU plus I^-, and ITU revealed complete degranulation of the *beta* cells (as observed in sections stained with Gomori's aldehyde-fuchsin) and hypertrophy, hyperplasia, and vacuolation of these elements. *Alpha* cell (acidophil) degranulation was complete, however, *only in those rats receiving PTU or PTU plus* I^-. This phenomenon of complete degranulation of the alpha cells has been interpreted to indicate an essentially complete inhibition of thyroid hormone synthesis (Griesbach and Purves, 1945; Goldberg and Chaikoff, 1949, 1950). In the 0.2 %ITU treated rats, the alpha cells were not only well granulated, but in five of the six were essentially normal. This finding probably indicated, therefore, that thyroid hormone synthesis was not completely blocked.

Another striking difference observed in ITU treated rats as compared with rats totally deprived of thyroid hormone is clearly brought out in Figure 1. It will be seen that the growth of PTU treated rats plateaus in about one month (as is the case with surgically or I^{131}-thyroidectomized rats;

Goldberg *et al.*, 1950); iodide administration does not alter the growth deficit. The administration of 0.16% ITU, however, results in a growth rate less than that of the control rats but greater than that of rats in which thyroid hormone synthesis is totally suppressed; in addition, no plateau is attained in this time interval.

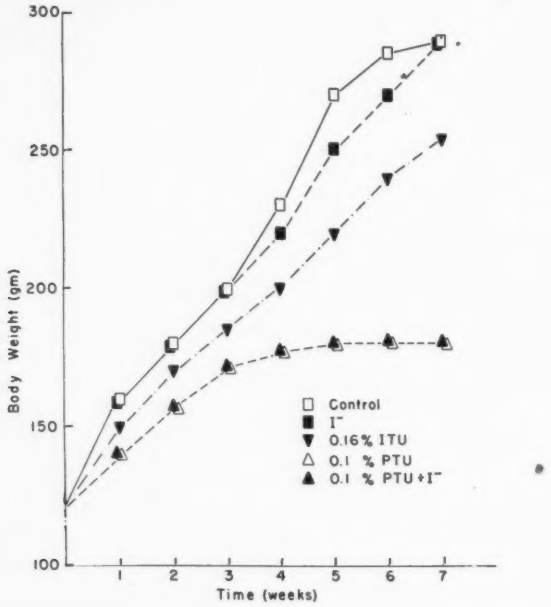

FIG. 1. Growth of rats maintained for seven weeks on various diets. Iodide added to diets is equimolar to that contained in 0.1% ITU. Each point represents the average weight of seven rats.

II. *Metabolism of P^{32} by ITU and PTU treated rats*

Because of the magnitude of *in vivo* hydrolysis of ITU with release of free iodide (Plummer, 1950) the problem of isotope dilution complicates the use of I^{131} for metabolic studies. Therefore, without knowledge of specific activities, quantitative evaluation of radioactive iodine uptake data is scarcely possible, although this has been attempted in two previous studies (Williams *et al.* (1949); Gassner *et al.* (1950)).

The studies of Borell and Holmgren (1949) indicated that the uptake of P^{32} by the guinea pig thyroid was a far more sensitive index of the degree of thyrotrophic stimulation than were acinar cell height measurements. In a preliminary study in the rat we observed that the injection of TSH[4]

[4] Kindly supplied by Dr. Irby Bunding of the Armour Laboratories, Chicago, Illinois.

TABLE 2. P^{32} UPTAKE BY THE THYROID GLAND OF RATS TREATED WITH ITU AND PTU

Expt.	Group	Treatment	Duration	Interval sacrificed after P^{32} injection	P^{32} uptake by thyroid gland
			days	hours	cts./min./mg.*
I	A	0.1 % PTU (6)†	2	1	147 ± 15
	B	0.16% ITU (6)	2	1	108 ± 14
	C	0.1% PTU plus I^- (6)	2	1	132 ± 15
	D	I^- (6)	2	1	99 ± 18
	E	Control (6)	2	1	107 ± 15
II	A	0.1% PTU (6)	4	1.5	202 ± 64
	B	0.16% ITU (6)	4	1.5	183 ± 51
	C	0.1% PTU plus I^- (6)	4	1.5	223 ± 31
	D	0.1% PTU plus 0.16% ITU (6)	4	1.5	200 ± 26
	E	I^- (6)	4	1.5	118 ± 15
	F	Control (6)	4	1.5	129 ± 32

* Mean ± Standard Deviation.
† Number of rats/group.

(Armour R 377158) (approximately 1 USP unit subcutaneously twice daily for 2 days) resulted in a two fold increase in the uptake of P^{32} by the thyroid 1 to 2 hours after P^{32} injection. As can be seen from Table 2, the administration of PTU for 2 or 4 days resulted in a slightly increased P^{32} uptake which was not modified by iodide ion. ITU seemed to exert less effect on P^{32} uptake but it is clear that the insensitivity of this method plus the large individual variations precluded accurate evaluation of thyroid activation.

III. *The effect of high doses of ITU on the rat thyroid and pituitary glands and on growth rate*

Since the above experiments suggested that 0.16 or 0.2% ITU administration was, perhaps, only partially as effective in blocking thyroid hormone synthesis as PTU, it was deemed of interest to investigate the effect of administering larger quantities of this compound. The effect of higher doses of ITU on growth rate, thyroid size, and pituitary cytology were compared with the effects of PTU administered in concentrations known to block completely thyroid hormone synthesis.

In Table 3 are shown the results of an experiment in which rats were fed a diet containing 1.0% ITU for one month. Note that the thyroid glands underwent hypertrophy to the same extent as did those of rats receiving 0.1% PTU (with or without iodide added on an equimolar basis). The thyroids from the ITU treated rats were grossly hyperemic, had massively hypertrophied isthmi, and bled freely on cut section. Microscopic exami-

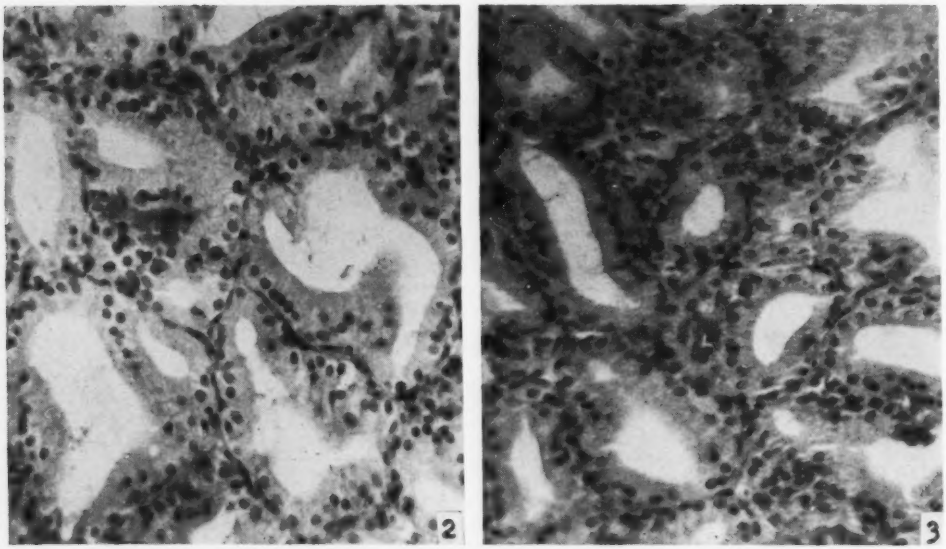

FIG. 2. Thyroid gland from rat maintained on a diet containing 1.0% ITU for one month. Note the complete absence of follicular colloid, extreme increase in acinar cell height, papillary infolding, and good vascularity. Hematoxylin and eosin; ×450.

FIG. 3. Thyroid gland from rat maintained for one month, on a diet containing 0.2% PTU. Note the similarity to the gland pictured in Fig. 3. Hematoxylin and eosin; ×450.

nation revealed a picture indistinguishable from that found in thyroids of rats which received PTU, i.e. almost complete absence of follicular colloid, extreme hypertrophy and hyperplasia of the parenchymal elements, intense vascularity, and a high degree of mitotic activity (Figs. 2 and 3). Cytological examination of the pituitary glands of the ITU treated rats revealed beta cell degranulation, hypertrophy, hyperplasia, and vacuolation, and *complete alpha cell degranulation;* an appearance indentical to that noted in the pituitaries of the 0.1% PTU treated rat, and indicative of total block of thyroid hormone synthesis.

It will also be seen from Table 3 that the addition of 0.16% ITU to a diet containing 0.1% PTU was no more effective in inhibiting the hypertrophy of the thyroid than was the addition of an equivalent amount of iodide to PTU. Findings of a similar nature were observed by Goldberg and Chaikoff (unpublished data, 1951) who observed that the administration of 0.2% ITU in the diet did not inhibit the thyroid gland response to *exogenous* TSH[5] (2 USP units daily for three days) as measured by gland weight and acinar cell height response.

[5] Kindly supplied by Dr. Wayne Donaldson of Parke Davis and Co., Detroit, Michigan.

TABLE 3. THE EFFECT OF HIGH DOSAGES OF ITU ON THE THYROID AND PITUITARY GLANDS

Expt.	Group	Treatment	Duration	Thyroid gland wt.	Histology	
					Thyroid	Pituitary
			weeks	mg./100 gm.		
I	A	0.1% PTU (7)*	4	27 ± 4†	H. and H.‡	Beta cells: hypertrophy, hyperplasia, degranulation Acidophils: total degranulation
	B	0.1% PTU plus I^- (6)	4	23 ± 7	H. and H.	Same as A
	C	I^- (5)	4	7.9 ± 0.9	normal	normal
	D	1.0% ITU (7)	4	33 ± 3	H. and H.	Same as A
	E	Control (7)	4	7.2 ± 0.8	normal	normal
II	A	0.1% PTU (6)	4	29 ± 3	H. and H.	Same as A (Expt. I)
	B	0.1% PTU plus 0.16% ITU (5)	4	20 ± 2	H. and H.	Same as A
	C	0.1% PTU plus I^- (6)	4	18 ± 3	H. and H.	Same as A
	D	0.16% ITU (7)	4	9.7 ± 1.5	normal	Beta cells: hypertrophy, hyperplasia, degranulation Acidophils: normal to slight degranulation
	E	Control (5)	4	6.3 ± 0.5	normal	normal

* Number of rats/group.
† Mean ± Standard Deviation.
‡ Hypertrophy and hyperplasia, colloid loss, high vascularity.

In contrast to the growth curves found when 0.16% ITU was administered (Fig. 1) it will be seen from Figure 4 that the administration of 1.0% ITU in the diet results in a growth curve identical to that obtained with TU administration.

IV. *Studies on the rate of release of thyroidal I^{131}: the effect of low and high concentrations of ITU and the failure of ITU to alter the acceleration of the rate of release effected by exogenous TSH*

The rate of release of thyroidal organic I^{131} has been shown by Wolff (1951) to be a reliable index of the rate of thyroid activity. ITU was as effective as 0.1% TU in accelerating the release of thyroidal organic I^{131} *only* when it was administered at the 1% level (Table 4), a dose level which is goitrogenic, However, when administered at the 0.2% level (approximately that used by previous investigators), ITU effected only a slightly greater rate of release of thyroidal I^{131} than was observed in the control rats.[6]

[6] It must be pointed out that, in view of the studies of Roche and co-workers (1952) and of Stanbury *et al.* (1953), interpretation of the quantitative significance of the release of thyroidal I^{131} is complicated by 1) the interference by antithyroid drugs with

TABLE 4. INFLUENCE OF ITU ON THE RATE OF RELEASE OF THYROID ORGANIC I^{131}

Expt.	Treatment†	Number of rats/gp.	Half life of thyroidal I^{131}
			days
I	Control	8	1.7
	I^-	6	1.7
	0.2% ITU	7	1.5
	1.0% ITU	8	0.9
	0.1% TU	7	0.7
	0.1% TU plus I^-	7	0.9
II	Control	6	2.0
	TSH*	7	0.7
	TSH plus I^-	5	0.6
	TSH plus 0.2% ITU	7	0.8
	TSH plus 0.1% TU	8	0.5
	TSH plus 0.1% TU plus I^-	6	0.5

* 10 USP units of TSH twice daily.
† Iodide added to diets in Expt. I is equivalent to that contained in 0.0% ITU, equivalent to 0.2% ITU in Expt. 2.

As an additional method of evaluation of any possible anti-TSH activity, ITU was administered to rats injected twice daily after the zero time count with 10 USP units of TSH[5] (Parke Davis preparation 50P4). It is evident from Table 4 (Experiment II) that there was no inhibition of the accelerated rate of release effected by TSH administration.

V. *The effect of ITU on the iodine content of the thyroid gland*

It has been reported by Barrett *et al.* (1951) that the administration of ITU results in a smaller decrease in thyroid iodine content than that observed in TU treated rats. In the present study male Sprague-Dawley rats were treated for two weeks with both low and high levels of ITU. It will be seen from Table 5 that not only is there no decrease in the thyroid (total) iodine content in rats treated with 1% ITU, but there is actually a significant increase. It is of importance to note, however, that in these rats, the content of *organic* iodine is reduced to the same level as that found in rats treated with 0.1% TU. An appreciable, although reduced, amount of *organic* iodine is still found in the thyroids of rats treated with 0.2% ITU.

VI. *Duplication of the "ITU effect" by simultaneous administration of TU and I^-*

It seemed clear from the above experiments that the apparently anomalous effect reported by previous investigators could probably be ascribed

the re-utilization of I^{131} hydrolyzed from monoiodo- and diiodo-tyrosine, and 2) by dilution of I^{131} by any I^{127} that may have been formed from the hydrolysis of ITU.

TABLE 5. EFFECT OF ITU AND TU ON THYROID I^{127} CONTENT

Treatment (22 days)	Number of rats/ group	Initial body wt. (avg.)	Final body wt. (avg.)	Thyroid gland iodine			
				TCA Soluble	Organic	Total	Per cent TCA soluble
		gm.	gm.	gamma	gamma	gamma	%
Control	7	172	255	0.6	12.5	13.1	4.6
0.1% TU	7	155	182	0.6	0.3	0.9	66.7
0.2% ITU	7	164	226	14.5	2.9	17.4	83.1
1.0% ITU	7	170	200	22.8	0.3	23.1	98.9
0.1% TU plus I^-	6	160	182	21.9	0.2	22.0	99.2

to either low molar antithyroid activity and/or TU formed by hydrolysis with modification of its goitrogenicity by iodide ion. In order to test further this possibility an attempt was made to duplicate this "phenomenon" with TU and I^-. Consequently, rats were placed on a diet containing 0.025% TU with or without equimolar iodide. As can be seen from Table 6, the goitrogenic activity of this dose of TU is almost completely reversed

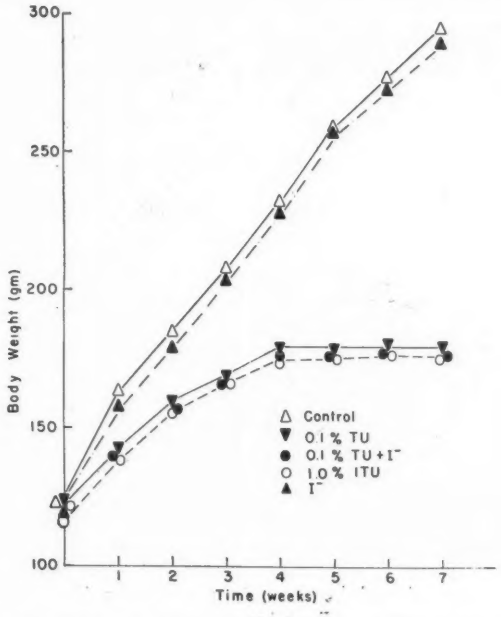

FIG. 4. Growth of rats maintained for seven weeks on various diets. Iodide added to diets is equimilar to that contained in 1.0% ITU. Each point represents the average weight of four to seven rats.

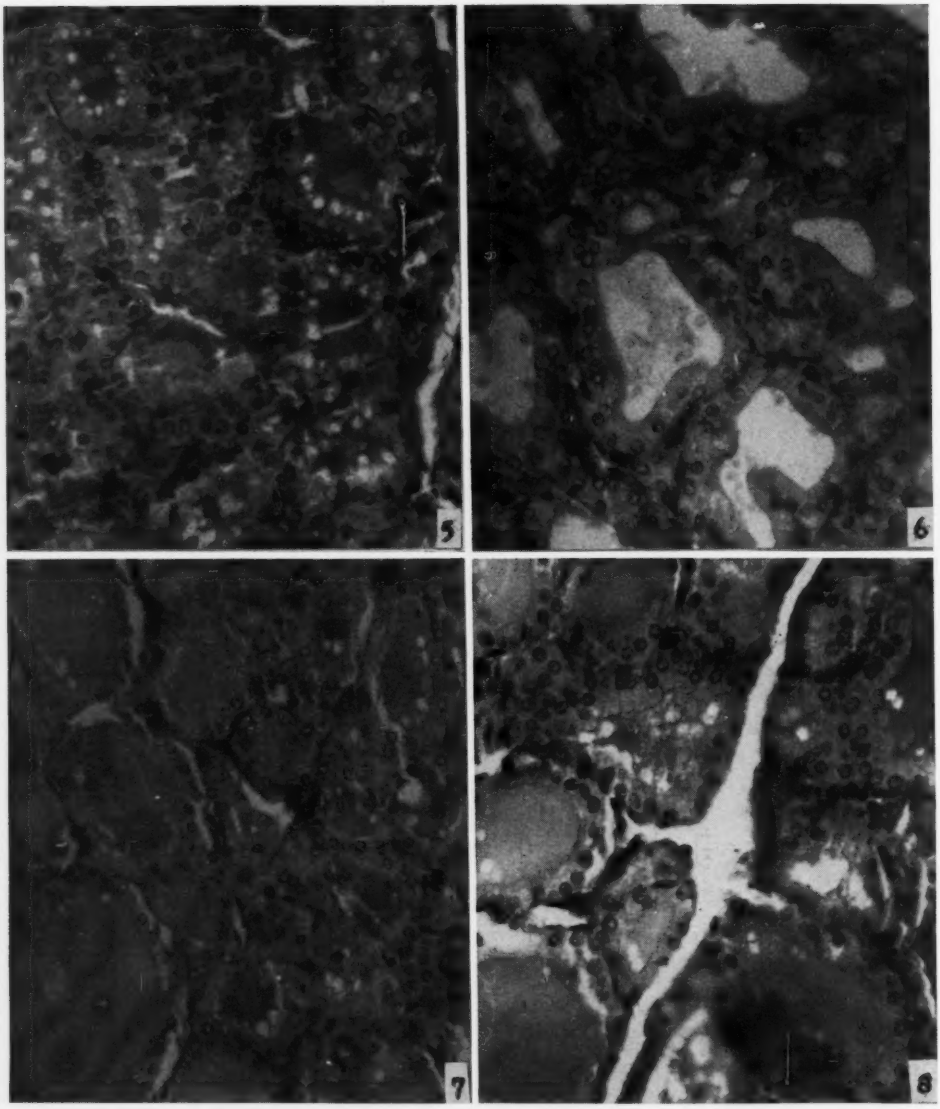

FIG. 5. Thyroid gland of rat fed control diet (no antithyroid agent or idodide added). The typical state of apparent a slight hyperactivity is illustrated by the relatively high (cuboidal to low columnar) acinar cells and the marked vacuolation of the colloid. Hematoxylin and eosin; ×500.

FIG. 6. Thyroid gland from rat fed a diet containing 0.025% TU for one month. The intense degree of activation is indicated by the almost complete absence of follicular

TABLE 6. COMPARISON OF LOW DOSES OF TU PLUS IODIDE WITH ITU AS TO EFFECT ON THE THYROID AND PITUITARY GLANDS

Group	Treatment (30 days)	Thyroid gland		Histology	
		Weight	Per cent of control	Thyroid gland	Pituitary gland
		mg./000 gm.	%		
A	Control (6)*	6.5 ± 1.5†	100	normal	normal
B	0.025% TU (7)	11.5 ± 1.0	177	H. and H.‡	Beta cells: hypertrophy, hyperplasia, degranulation Acidophils: slight to insignificant degranulation
C	0.025% TU plus I^- (7)	7.3 ± 1.6	112	normal	same as B
D	0.16% ITU (7)	7.7 ± 1.3	119	normal	same as B
E	I^- (6)	6.4 ± 0.5	99	normal	normal

* Number of rats/group.
† Mean ± Standard Deviation.
‡ Hypertrophy and hyperplasia, colloid loss, high vascularity.

by the addition of iodide. Histological examination of the thyroids of the rats treated with TU revealed an intense hypertrophy with hyperplasia of the parenchymal elements, nearly complete absence of follicular colloid, and a very high degree of vascularity (Fig. 6). Administration of iodide (Fig. 7) reversed almost completely the histological signs of activation. These glands, as those from the ITU treated rats (Fig. 8), were readily distinguishable from the controls which, as can be seen in Figure 5, reveal indications of moderate activity.

Cytological examination of the pituitary glands revealed a similar picture in the three experimental groups receiving an antithyroid agent, i.e., hyperplasia, hypertrophy and degranulation of the beta cells, and slight to insignificant degranulation of the alpha cells (Table 6). This, as

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colloid, tall columnar hypertrophied acinar cells, papillary infolding, and hyperemia (erythrocytes appear black in the dilated peri-acinar capillaries). Hematoxylin and eosin; ×500.

FIG. 7. Thyroid gland from rat fed a diet containing 0.025% TU and iodide (equimolar) for one month. Note that the follicles are well filled with colloid, the flattening of the epithelium, reduction in vascularity, and inhibition of cellular hypertrophy and hyperplasia as compared to the thyroid shown in Fig. 6. Note, however, that areas revealing both activation and inactivation can be found in the same field. Hematoxylin and eosin; ×500.

FIG. 8. Thyroid gland from rat fed a diet containing 0.16% ITU for four weeks. The thyroids, as typified by this picture, were histologically not distinguishable from those of the TU plus iodide group. The latter two groups had thyroids which were histologically somewhat less active than those of the controls. Hematoxylin and eosin; ×500.

pointed out earlier, is evidence of incomplete suppression of thyroid hormone synthesis.

The above findings demonstrate, therefore, that weak antithyroid activity without marked goitrogenicity may result from the combined action of the breakdown products of ITU (TU and I^-; Plummer, 1950) and that the integrity of the ITU molecule is not an indispensable requisite in the elucidation of its "anomalous" actions.

DISCUSSION

Astwood's (1945) early studies of thiouracil derivatives had shown that the 5-polar substituted thiouracils possessed little if any goitrogenic action in rats. Thus, the 5-cyano- and 5-carbethoxy-congeners were *not goitrogenic* at concentrations of approximately 1% in the diet. The obvious implication was that these compounds were, therefore, *not antithyroid;* and the studies of Gassner *et al.* (1950) claiming antithyroidal action without goitrogenicity for another 5-polar substituted thiouracil (ITU) were hence received with some surprise.

It is clear from the results of the present study that the apparently anomalous ability of ITU to effect a separation between antithyroid action and goitrogenicity is not manifested when the dosage of administered drug is raised to sufficiently high levels. Thus, markedly goitrous, histologically active thyroid glands consistently obtain in rats maintained on a diet containing 1% ITU. Growth of these animals was arrested to the same extent as in animals receiving goitrogenic doses of PTU or TU. It was only in such glands (from animals treated with 1% ITU) that the *organic* iodine content was reduced to a level comparable to that obtained with with a known potent antithyroid agent. Moreover, an accelerated rate of release of thyroidal I^{131}, comparable to that obtained with thiouracil, occurred only with the administration of 1% ITU. It is of interest to note, in this connection, that Albert and Ford (1952) administered ITU at a level of 1% in the diet and observed a similar augmentation of the rate of biological decay of thyroidal I^{131}.

In discussion of their experiments Gassner *et al.* (1950) suggested that perhaps ITU interfered with the action of thyrotrophic hormone on the thyroid gland. Since in the present experiments ITU 1) is no more effective in inhibiting the goitrogenic action of propylthiouracil than is an equivalent amount of iodide; 2) is markedly goitrogenic when administered alone; 3) does not inhibit the increased rate of release of thyroidal I^{131} effected by exogenous TSH; and since it does not inhibit the increased histological activity of the thyroid gland of rats treated with exogenous TSH, it may be assumed that some more feasible explanation of the earlier experiments is possible.

It is difficult to ascertain to what extent the thyroid depression is mediated by the intact molecule and/or by free TU released during *in vivo* hydrolysis. It has been reported by Plummer (1950) that, in the dog, up to 50% of the drug excreted in the urine appears as the hydrolyzed product.

It was shown that goiter, resulting from the administration of low dosages of TU could be almost completely suppressed by iodide ion. Similar experiments have already been reported by Mackenzie (1947) and by Kjerulf-Jensen (1946). Since dose levels (0.16 or 0.2%) of ITU which were minimally goitrogenic presented cytological, respiratory, growth, and chemical evidence of partial suppression of thyroid hormone synthesis, it is possible that the "non-goitrogenicity" of ITU may be due to the combined effect of weak antithyroid activity (ITU *per se* and/or thiouracil released in relatively low concentrations) modified by iodide released in the hydrolysis of ITU. However, when ITU is administered in high enough concentrations (1%), thyroid hormone synthesis can be essentially completely blocked, and at this dose level ITU is no less goitrogenic than PTU or TU.

The evaluation of ITU as a clinical agent of importance in the therapeutic and preoperative management of thyrotoxicosis has led to somewhat divergent opinions (Williams *et al.*, 1949; McClintock and Lyons, 1951a, 1951b; Bondy, 1951; Starr and coworkers, 1951, 1952, 1953; Goldner and Kirschenfeld, 1952; Bartels, 1952; and Seven and Perloff, 1953). In the treatment of thyrotoxicosis one must remember that, in addition to the direct antithyroid activity of the thiouracil moiety, iodide *per se* inhibits the hyperactive gland and the clear separation of these two activities is difficult. Because of the rapidity of depression of metabolic rate, it would seem, that at least initially, one is dealing with an iodide effect, and it is interesting to speculate as to whether one of the functions of ITU might not be to act as a depot for a steady release of iodide at high levels.

SUMMARY

Experiments designed to test the antithyroid potency of iodothiouracil (ITU), reputed to be a non-goitrogenic antithyroid agent, revealed that it is relatively weak, as compared on a molar basis to propylthiouracil (PTU) or thiouracil (TU) in the rat. When administered at concentrations at which its goitrogenic action is minimal (approximately that level used by previous investigators) it fails to suppress thyroid hormone synthesis completely as judged by 1) QO_2 of liver and kidney slices; 2) presence of reduced but appreciable concentrations of *organic* iodine in the thyroid gland; 3) inability to degranulate totally the alpha cells of the anterior pituitary gland; 4) incomplete growth suppression and 5) only slight acceleration of the rate of release of thyroidal I^{131}.

Administration of high levels of ITU (1% in the diet) results in marked goitrogenicity and evidence, from the above criteria, of essentially complete suppression of thyroid hormone synthesis.

No evidence was found to indicate that ITU inhibits either the release of TSH or its action in stimulating the thyroid gland.

In contrast to recent reports the administration of ITU not only does not decrease the total iodine content of the thyroid gland but actually results in an increase. When goitrogenic levels of ITU are administered the organic iodine content is reduced to a level comparable to that seen with the administration of PTU or TU.

Effects similar to those observed with non-goitrogenic doses of ITU were duplicated, in part, by the simultaneous administration of low doses of TU (0.025% in the diet) and equimolar iodide ion (0.022%).

P^{32} uptake by the rat thyroid gland was found to be a relatively insensitive criterion for extimation of TSH stimulation of the thyroid; its usefulness as an assay procedure in this species appears quite limited.

Acknowledgments

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BIOASSAY OF ADRENAL CORTICAL STEROIDS ON THE BASIS OF ELECTROLYTE EXCRETION BY RATS: EFFECTS OF 11-DESOXY and 11-OXY-STEROIDS[1]

BEN B. JOHNSON

Department of Medicine, Stanford University School of Medicine, San Francisco, California

THERE has been a recent increase of interest in the measurement of electrolyte activity of the adrenal cortical steroids. Studies have been made not only on the role of the known steroids in fluid and electrolyte regulation, but also on the question of what electrolyte-active compounds are secreted by the adrenal cortex and appear in blood and in urine.

Methods of bioassay, based on the effects of steroids on sodium and potassium excretion, have been used in the detection of a highly active corticoid in adrenal cortical extract and adrenal vein blood (Grundy, Simpson, Tait, and Woodford, 1952). A similar corticoid has been found to appear in unusual quantity in the urine of certain patients with edema (Luetscher and Johnson, 1953). The increased sodium-retaining activity of urine extracts from these edematous patients has been observed to decrease when diuresis was induced (Luetscher and Deming, 1950; Luetscher, Deming and Johnson, 1951). Further investigation will be necessary to evaluate the importance of such adrenal cortical secretions in the regulation of electrolyte balance.

Dorfman (1953) has recently reviewed the results, precision, and limitations of the sensitive methods for assay of desoxycorticosterone and some related compounds. The present report includes an examination of certain aspects of experimental design and calculation of results by which the assay can be directed to answer specific questions. The method presented allows a qualitative distinction between the effects of 11-desoxy and 11-oxycorticosteroids, as well as a quantitative estimate of desoxycorticosterone-like effects.

METHOD OF ASSAY

For each assay nine female albino rats of the Slonaker strain (Stanford colony, inbred since 1924) weighing 150 to 160 grams, are used. Bilateral adrenalectomy is performed

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4 days before the assay. For the next 3½ days the rats are maintained on stock diet (Addis and Gray, 1950) and 0.9% saline to drink. At 11 A.M. one day before the start of assay they are given 3.3 ml. distilled water by intraperitoneal injection. Starting at 4 P.M. on this day, sodium intake is strictly controlled. The rats are allowed only sodium-free stock diet and distilled water, and are given an intraperitoneal injection of 5 ml. 0.9% NaCl solution in the late afternoon before each assay day.

The solvent for materials tested is 95% ethyl alcohol, which has been purified either on a column of activated charcoal or by distillation after the addition of lead acetate and potassium hydroxide (USP XIII). The standard is an 11-desoxycorticosterone acetate solution in 95% ethanol containing 5 μg. in the injected volume of 0.05 ml. The material to be assayed is dissolved in 95% ethanol so that the desired individual dose is contained in 0.05 ml. The nine rats for each assay are subdivided into three groups of three rats, not allowing litter mates within groups. The DOCA standard (S), test material of undetermined potency (U), and solvent control (C) are given to each group in a different order on three successive days, as suggested by Spencer (1950), according to the following plan:

Group I—1st day C, 2nd day S, 3rd day U;
Group II—1st day U, 2nd day C, 3rd day S;
Group III—1st day S, 2nd day U, 3rd day C.

On the morning of each assay day, each rat receives by intramuscular injection control, standard, or unknown as determined in the above plan. An intraperitoneal distilled water load of 5 ml. is then given, and the rat is placed in an individual metabolism cage for collection of urine over the next 5 hours. At the end of the collection period, inhalation of ether is used to stimulate bladder emptying. Variation in timing is minimized by taking each rat in the same order for all procedures.

The urine is collected in a graduated centrifuge tube and at the end of the collection period the glass funnel and collecting apparatus for each rat are rinsed with 5 ml. distilled water. The following measurements are made for each rat on each of the 3 days: urine volume, final volume after rinsing, urine sodium, and urine potassium. Flame photometry is used to determine sodium and potassium. Each rat is weighed at the start of the first assay day and after urine collection on the last day, and is followed subsequently for possible survival on stock diet and tap water to drink.

A rat is discarded from an assay for any of the following causes: Urine volume less than 0.3 ml. on any day; total urine sodium less than 30 microequivalents on the control day; survival after withdrawal of saline at end of assay; obvious illness at any time, for example: severe diarrhea or hematuria, marked coldness, or inability to struggle during the ether inhalation. Male rats have been successfully used, but appear to show greater variability in all measurements and to tolerate the procedure less well.

RESULTS WITH DESOXYCORTICOSTERONE ACETATE

Fourteen assays have been done with four different doses of DOCA as the "unknown" material compared with solvent control and 5 μg. standard: three assays of 10 μg. DOCA, four assays of 5 μg., four assays of 2.5 μg., and three assays of 0 μg. (solvent injected). The results of these experiments have been subjected to statistical procedures, and form the basis for the present method of estimation of potency of unknown materials and of

standard error. These procedures have been carried out under the direction of Dr. Lincoln Moses of the Department of Statistics.

The response may be calculated on the basis of sodium output or of the ratio of potassium to sodium output. Either method may be more advantageous, depending on the unknown studied, as will be shown.

Homogeneity of data on sodium output and potassium-sodium ratio

Since it was suspected that observations with high sodium output were more variable than those with low sodium, six experiments were analyzed to investigate the degree of correlation between mean and variance. The experimental plan gives nine sets of three observations, e.g., 2nd day standard injection, first day control injection, etc. For each such set within an experiment the mean and variance of the observations were computed and Kendall's rank correlation coefficient (Kendall, 1948) was computed. This procedure was carried out for the data in their original form, as well as in the transformations to logarithms and to square roots. The results, Table 1, show striking association of high variability with high sodium output using the raw data. The log transformation tended to overcorrect, while the square root expression showed the least correlation of variability with high or low output.

For the case of potassium/sodium output ratio, the same experiments were similarly tested using the unmodified data, the log transformation, and the square root transformation (Table 1). Again, the unmodified ratios showed high correlation of variability with large numbers, but the square root expression corrected inadequately. The log K/Na was satisfactory. Accordingly, two analyses of variance were done for each of the fourteen experiments, expressing the individual observations both as the square root of total sodium output and as log total K/total Na output ratio.

TABLE 1. RANK CORRELATION TEST: SUMMARY OF τ VALUES*

Assay No.	Total Na	Log Na	$\sqrt{\text{Na}}$	$\frac{\text{Total K}}{\text{Total Na}}$	$\text{Log}\frac{\text{K}}{\text{Na}}$	$\sqrt{\frac{\text{K}}{\text{Na}}}$
4	+0.278	0.0	0.0	+0.389	0.0	+0.278
5	+0.667	+0.333	+0.389	+0.278	−0.389	−0.111
6	+0.389	−0.278	−0.111	+0.500	+0.222	+0.333
12	+0.278	−0.556	−0.278	+0.667	+0.333	+0.500
13	+0.389	−0.556	−0.056	+0.500	+0.222	+0.500
14	+0.333	−0.167	+0.167	+0.445	−0.333	+0.111
Totals	+2.334	−1.224	+0.111	+2.779	+0.055	+1.611

* τ = Kendall's rank correlation coefficient: A value of +1.00 representing the maximum possible positive correlation of high observed values with high variances. A coefficient of −1.00 indicates complete inverse ranking correlation between variance and size of observations. A value of 0 indicates no correlation, and suitable transformations of the data should give totals for the six experiments approximating 0.

Analysis of variance: square root sodium

Table 2 summarizes the analyses of variance for the fourteen experiments in terms of $\sqrt{Na}$. The experiments are arranged in increasing order of "unknown" dose. The mean squares from each experiment are shown. Bartlett's test of homogeneity of variances indicates that the error mean squares may be regarded as estimates of a common error variance; they

TABLE 2. ANALYSIS OF VARIANCE FOR DOCA ASSAYS. DATA EXPRESSED AS $\sqrt{Na}$ OUTPUT

Assay	Dose of DOCA assayed	No. of rats	Mean squares				
			Between groups	Within groups	Days	Treatments	Error
1	0 μg.	9	2.77	12.16	10.06	22.93	1.929
2	0 μg.	8	3.99	3.33	26.01	26.05	1.363
3	0 μg.	9	7.15	14.72	5.93	18.93	2.433
4	2.5 μg.	9	6.82	5.02	0.06	2.99	3.153
5	2.5 μg.	8	0.72	4.72	4.16	16.77	3.726
6	2.5 μg.	7	0.98	5.71	0.78	4.76	5.228
7	2.5 μg.	8♂	11.38	8.89	6.17	40.73	2.409
8	5 μg.	8	11.42	9.03	6.32	13.83	2.875
9	5 μg.	9	8.06	5.45	9.58	5.65	6.511
10	5 μg.	7	11.21	19.71	3.76	49.53	2.661
11	5 μg.	9	7.30	5.22	2.47	18.61	2.578
12	10 μg.	9	14.80	8.52	2.53	36.43	2.491
13	10 μg.	9	14.42	4.41	2.42	37.01	2.809
14	10 μg.	8	2.37	1.62	10.71	33.35	1.926

Error variance of 14 assays pooled (178 degrees of freedom) = 2.995.

may thus be pooled, and the resulting estimate is 2.995. If the mean squares in the column headed "days" are compared to this figure, it is seen that there is no evidence of a consistent shift in general level of response on days 1, 2, and 3, but that in a few instances there was a marked day effect (assays 1, 2, 14). The entries in the column headed "within groups" reflect variability among rats subjected to the same order of doses; the fact that these run rather consistently greater than 2.995 indicates that the design chosen, which permits segregating variability among rats, has increased the precision of the experiments. Entries in the column headed "between groups" reflect differences among the three groups of rats in each experiment receiving the doses in one of the three orders used. If the order in which the doses were received greatly affected the response of the rats to any one or more doses this would tend to make the entries in this column larger than those in the "within groups" column. Inspection shows that if any order effect existed it was not large enough to manifest itself in this way. Entries in the column headed "treatments" reflect the degree to which the average responses to control, standard, and test injections

differed from one another. The size of the numbers appearing here should be related less to the absolute size of the "unknown" dose than to the spread among all three doses. Thus, one should expect the smallest values to be associated with 2.5 μg. and the largest with 10 μg. Comparison of the 14 numbers appearing in this column with the value 2.995 leaves little doubt that the animals gave convincingly different response to different doses of DOCA.

Analysis of Variance: log K/Na

Expressing the response in the same 14 standard assays as log K/Na output ratio, the corresponding data are summarized in Table 3 for comparison. The different variables are related in the same way as for the sodium

TABLE 3. ANALYSIS OF VARIANCE FOR DOCA ASSAYS

DATA EXPRESSED AS $\log \frac{K}{Na}+1.0$

Assay	Dose of DOCA assayed	No. of rats	Mean squares				
			Between groups	Within groups	Days	Treatments	Error
1	0 μg.	9	.0722	.0743	.0972	.3174	.0101
2	0 μg.	8	.0548	.0226	.0826	.2673	.0173
3	0 μg.	9	.0984	.1100	.0117	.2316	.0263
4	2.5 μg.	9	.0056	.0360	.1871	.1146	.0167
5	2.5 μg.	8	.0047	.0515	.1344	.2111	.0294
6	2.5 μg.	7	.0004	.0484	.1972	.1295	.0475
7	2.5 μg.	8♂	.0091	.0643	.2402	.2475	.0334
8	5 μg.	8	.0921	.0819	.0896	.1531	.0092
9	5 μg.	9	.1497	.0501	.0163	.1254	.0273
10	5 μg.	7	.0090	.1122	.0169	.3532	.0120
11	5 μg.	9	.1043	.0502	.0202	.2483	.0273
12	10 μg.	9	.0546	.1060	.0038	.6368	.0325
13	10 μg.	9	.1434	.1250	.0472	.8320	.0205
14	10 μg.	8	.04117	.0199	.0821	.4431	.0070

Error Variance of 14 assays pooled (178 degrees of freedom) = 0.02248.

data of Table 2, but the effects of treatment are considerably larger in proportion to error variance, and are in general more consistently related to dose. The mean squares for error are again found to be homogeneous, and are pooled to give a combined estimate of 0.02248. Being based on 178 degrees of freedom, this estimate of error should have considerable precision.

In order to compare the error of experiments in which the substance assayed was not a known dose of DOCA, the following procedure was employed: thirteen representative complete experiments with urine extracts

(whole or chromatographic fractions) as the unknown were chosen at random from different months and different ranges of potency throughout the year February, 1952, to 1953. The experiments were subjected to analylysis of variance expressing individual observations as log K/Na, and the mean squares for error were calculated. These mean squares for error were found to be homogeneous with each other, with a range from 0.0115 to 0.0411, and a combined error variance of 0.02394 with 182 degrees of freedom. These data are homogeneous with the corresponding findings in the standard experiments presented in Table 3. Assay calculations expressed as log K/Na are therefore considered to have known standard error.

Calculation of Dose-Response with DOCA

The conventional calculation methods such as those of Spencer (1950) and Singer and Venning (1953) have been to express response as the percentage decrease in control Na output, and to use a logarithmic dose scale. A different expression has been used in the present method in order to have a zero point on the dose scale and to distinguish assays in which the dose estimate has a high probability of being "less than zero," an effect produced by steroids such as hydrocortisone, which cause increased Na excretion.

For calculations based on log K/Na output, response (y_1) is expressed as average of log ratios for the unknown material being assayed, minus the average for the combined standard and control observations (Figure 1). In the graph are plotted the dose-response points for the 14 assays described previously in which the "unknown" was a dose of DOCA from 0 to 10 μg. The regression of response on dose for the 14 assays is indicated by the line with the equation given. Each point plotted represents the result of an entire assay. Approximate 95% confidence limits for estimation of dosage in a single 9-rat assay, calculated as the log K/Na ratio, and using the regression line defined by these 14 assays, is ±2.15 μg. DOCA.

The data from these same assays are similarly presented in Figure 2, using the square root calculation based on sodium output only. Here a positive response (y_2) is expressed by the average square root sodium for the combined standard and control observations minus the average for the material assayed. There is clearly a wider scattering of points about the regression line when only the sodium output is considered, and the calculated 95% confidence limits for an estimate of dose in a 9-rat assay are ±3.89 μg. DOCA.

Calculation of Assays with Rats Discarded

Using the criteria described under Methods, it is frequently necessary to eliminate one or more of the 9 rats from an assay. The most common

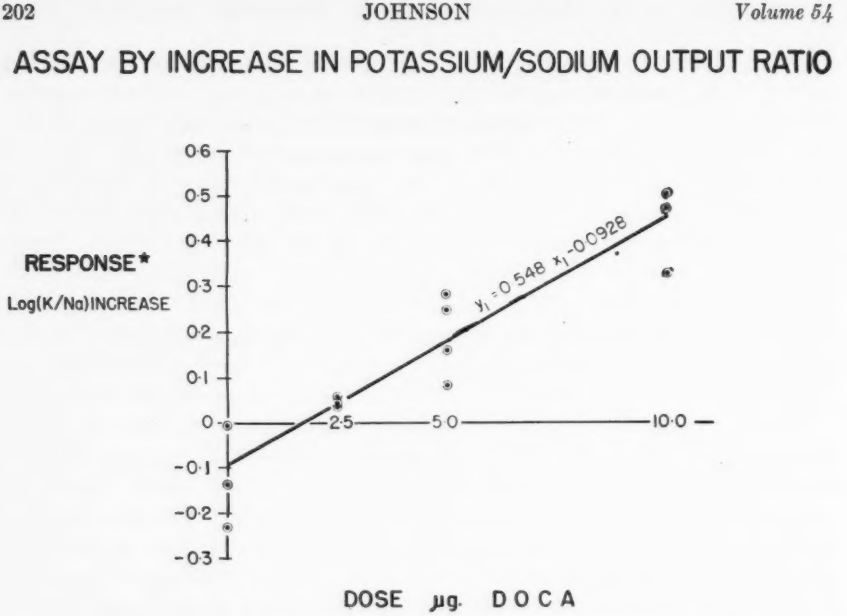

FIG. 1. *Response $(y_1) = U$ minus $(C+S)/2$. U = averaged log (K/Na) output ratios for each rat when the unknown was given, and C and S are the corresponding expressions for solvent control and 5 µg. DOCA standard.

cases are those in which one rat is missing from one group or one from each of two groups. In these cases calculation of average responses to a treatment as described earlier for complete 9-rat assays would give a biased estimate, because of the highly variable effect of the day on which treatment is given.

By statistical analysis it was possible to obtain unbiased mean square values for those standard experiments described in Tables 2 and 3 which lack one or two rats. Once the applicability of known-variance procedures was established for these assays, analysis of other experiments with discarded rats reduced to using appropriate formulas for unbiased estimation of treatment effects, at the cost of some loss in precision. Table 4 gives comparative confidence limits for the K/Na ratio and square root Na calculations of an assay when one or more rats must be discarded. In doubtful cases, the assays are repeated.

RESULTS WITH OTHER SODIUM-RETAINING CORTICOSTEROIDS

Other materials which decrease sodium output and increase potassium output can be related to DOCA by the calculations described. For example, Reichstein's Compound S in a dose of 200 µg. produces an effect on sodium

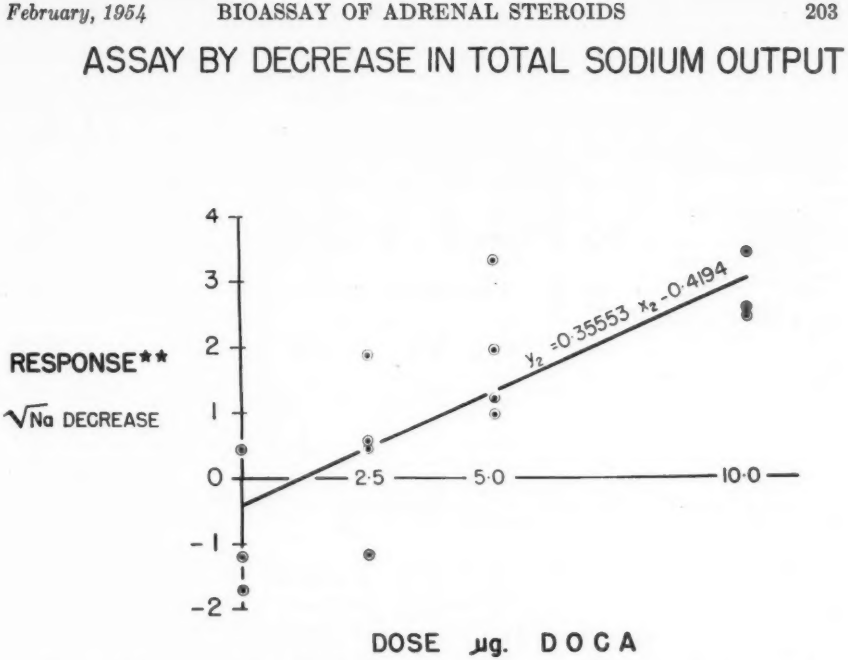

FIG. 2. **Response $(y_2) = (C+S)/2$ minus U. U = averaged square roots of the sodium output for each rat when the unknown was given, and C and S are the corresponding expressions for solvent control and 5 µg. DOCA standard.

output equivalent to 8.1 µg. of DOCA and an effect on log K/Na equivalent to 6.2 µg. DOCA, (Table 5). The response to 5 µg. free DOC is given for comparison. The effect of the unidentified sodium-retaining corticosteroid found in the urine of edematous patients has been assayed in a similar manner (Luetscher and Johnson, 1953).

RESULTS WITH 11-OXY-CORTICOSTEROIDS

When certain active 11-oxy-corticosteroids are assayed by the procedure described, the effects observed are qualitatively different. In ef-

TABLE 4. 95% CONFIDENCE LIMITS FOR ESTIMATE OF DOSE IN A SINGLE ASSAY

Number of usable rats	Dosage in 0 to 10 µg. range	
	log K/Na calculation	√Na calculation
9	±2.15 µg. DOCA	±3.90 µg. DOCA
8	2.34 µg. DOCA	4.16 µg. DOCA
7	2.51 µg. DOCA	4.46 µg. DOCA
6 (2 in each group)	2.68 µg. DOCA	4.77 µg. DOCA

TABLE 5. CONTRASTING EFFECTS OF 11-DESOXY AND 11-OXY CORTICOSTEROIDS

Compound	Dose	Assay: μg. DOCA equivalent		% Increase above control*		
		$\sqrt{Na}$	log K/Na	Na	K	Urine volume
11-desoxycorticosterone (DOC)	5 μg.	4.3 μg. ±3.9	3.3 μg. ±2.2	−36	3	− 8
11-desoxy-17α hydroxy-corticosterone (Reichstein's S)	200 μg.	8.1 μg. ±3.9	6.2 μg. ±2.2	−63	11	−16
17-hydroxy-corticosterone (Hydrocortisone) acetate	50 μg.	"−11.6 μg." ±4.5†	"−2.3 μg." ±2.5	113	55	45
11-dehydro-17-hydroxy-corticosterone (Cortisone) acetate	112 μg.	"−6.9 μg." ±4.2	"−1.6 μg." ±2.3	39	22	21
11-dehydro-corticosterone (Kendall's A) acetate	168 μg.	"−7.1 μg." ±3.9	"−1.0 μg." ±2.2	47	39	59
Hydrocortisone acetate DOCA	50 μg. mixture 5 μg.	"−4.5 μg." ±3.9	2.1 μg. ±2.2	10	65	61

* Ratios of test dose/control averaged as logarithms.
† Calculated values less than 0 μg. are given for comparison, enclosed in quotation marks. They can not be literally interpreted as negative DOCA equivalents; their 95% confidence limits are given only to illustrate significant departures from 0 dose, and have no other established quantitative meaning.

fective dosage, these steroids produce increased excretion of sodium, potassium, and water (Tables 5 and 6). Increases of 30% or more above the control output have been regarded as probably significant. The following doses produced definite effects: hydrocortisone 50 μg., cortisone 112 μg., corticosterone 100 μg., and 11-dehydro-corticosterone 168 μg. Hydrocortisone was considered the most potent of this group and was shown to be more effective than cortisone or corticosterone when compared in the same rats.

Calculation of response

If these 11-oxy-corticosteroids are compared with a DOCA standard, calculation on the basis of the square root of sodium output yields a dosage estimate less than zero (negative μg. DOCA effect). This expression can be used to indicate whether the response is below the 95% confidence limits for a control injection (Table 5).

Calculation on the basis of the log K/Na ratio may yield an estimate indistinguishable from zero, even when large changes are observed (Table 5). Since no adequate expression of 11-oxy-corticosteroid effect is possible with the usual DOCA standard, a hydrocortisone acetate standard has been used in a few experiments for measurement of comparative effect. Dose-response curves have not been established. For the 11-oxy-corticosteroids found to be active, Table 6 gives changes in sodium, potassium, and water excretion compared to the control observations in the same rats.

A mixture of effective doses of a sodium-retaining and an 11-oxy-steroid, as shown in Table 5 for DOCA and hydrocortisone acetate, may

TABLE 6. EFFECTS OF 11-OXYSTEROIDS AND OF 17-HYDROXY-11-DESOXYCORTICOSTERONE ON OUTPUT OF SODIUM AND POTASSIUM AND ON URINE VOLUME

Substance injected	Dose μg.	Expt. No.	No. of rats	% Increase above control*		
				Na	K	Volume
Hydrocortisone						
F acetate	12.8	1	7	45	—	39
F acetate	22.8	2	7	11	—	45
F acetate	50	4	8	30	39	76
F acetate	50	—	7	113	55	45
F acetate	100	3	9	49	45	47
F	100	—	7	33	—	75
Cortisone						
E	2	7	13	−4	−10	−7
E	5	7	13	18	9	3
E	5	8	7	1	18	21
E acetate	12.7	1	7	−8	—	−15
E	20	8	7	3	21	26
E acetate	22.7	2	7	1	—	33
E acetate	112	—	8	39	22	21
E acetate	112	3	9	64	0	21
11-Dehydro-corticosterone						
A acetate	50	—	9	−7	16	3
A acetate	168	—	9	47	39	59
A acetate	490	—	7	100	34	19
Corticosterone						
B	10	5	9	19	9	45
B	100	5	9	30	33	56
B	100	4	8	27	16	25
B	400	5	9	61	89	156
17-OH-11-Desoxy-corticosterone						
S	10	6	11	−16	5	2
S	100	6	11	−35	24	18
S	400	6	11	−58	8	5

* Ratios of test dose/control averaged as logarithms. Doses followed by the same experiment number were compared in the same rats.

have no very striking effect either on $\sqrt{Na}$ or log K/Na responses while a marked effect is revealed by the potassium and water output data.

DISCUSSION

The assay technique described was developed for measurements of steroids in biological fluids. Since such steroids may occur as mixtures and in limited amounts, a suitable method should distinguish different kinds of activity and have as much sensitivity and economy of material as possible.

In a recent review of assays based on electrolyte effects, Dorfman (1953) has considered the sensitivity and variability of the reported methods. The present report indicates the relative importance of certain factors in the experimental design and calculation of results, which should be taken

into account in the selection of an assay to deal with a specific problem.

The use of adrenalectomized animals is important. When adrenalectomy is found to be incomplete, the rat's output of sodium is low and insensitive to treatment. Although a theoretical objection to the use of adult female rats is the potential variability of the effect of the ovarian steroids, female rats have shown no greater variability and have tolerated the experimental procedure better than the males.

The observed variability between groups of rats is considerable. The use of each rat as his own control minimizes this error in the measurement of response (Spencer, 1950). Calculation of 95% confidence limits for estimates of dose confirms the estimate by Dorfman (1953) of the larger number of animals needed for an assay of greater precision. The use of 9 rats for three observations in the present method allows some economy in the number of animals used.

Dorfman, Potts, and Feil (1947) pointed out the sensitivity of the assay of desoxycorticosterone in adrenalectomized rats, using as a measure the retention of radioactive sodium by the rats. The procedures of Spencer (1950), of Singer and Venning (1953), and of Kagawa, Shipley, and Meyer (1952) also depend on diminished excretion of sodium. Previous reports from this laboratory (Deming and Luetscher, 1950; Luetscher and Deming, 1951), based on the output of sodium, have stressed the importance of strict control of the rats' sodium intake. Even with this precaution, the variability of sodium output has been large, especially in the high output observed after control or inert injections. When the square root of the sodium output is taken, the variability of these large outputs is reduced to a level comparable to that of the smaller outputs observed after injection of DOCA or similar materials. A dose-response relationship of uniform variance can then be constructed. In spite of the considerable variability, this measurement is useful in distinguishing the direction of effect on sodium excretion. An assay based on sodium output has certain obvious fallacies since the measurement may reflect failure of sodium excretion in a sick rat, or after injection of toxic material. Since the effect of the solvent used as a vehicle may be appreciable, no modification of strict solvent control is allowable.

Simpson and Tait (1952) have used the ratio of sodium to potassium excretion as a measure and have indicated that this expression minimizes certain technical errors. The use of the ratio between potassium and sodium excretion in the present technique has been demonstrated to reduce the variability of the response. This appears to be due in part to an increase in potassium excretion at doses above 5 μg. DOCA and in part to a tendency of the output of potassium to parallel the output of sodium

when the variation is related to non-specific changes. In this way, a change in K/Na ratio is a more selective test for DOCA effect than the sodium output alone. When the logarithm of the ratio is used, the closer confidence limits allow an estimate of dose nearly twice as precise as that based on sodium alone.

The 11-oxy-corticosteroids tested in effective dosage produced a clear-cut increase in the excretion of sodium and of water. These results are more clearly shown in this technique, in which a large water load is given at the beginning of the test, than in other reported techniques using loads of small volume or of isotonic saline solution. Dorfman (1949) reported no effect on sodium excretion when several 11-oxy-steroids were tested, except for increased sodium excretion when hydrocortisone was given in a dose of 50 μg. Marcus, Romanoff, and Pincus (1952) described small decreases in sodium excretion at 10 μg. doses of the 11-oxysteroids and small increases in sodium excretion at a dose of 200 μg. Singer and Venning (1953) found mild to marked sodium-excreting effects after doses of cortisone from 1 to 52.5 μg.

The present data demonstrate increases in potassium excretion after administration of the 11-oxy-corticosteroids, similar to those described by Marcus, Romanoff, and Pincus (1952). This parallel effect on sodium and potassium output makes the expression of the results as a ratio appropriate only if detection of 11-oxy-corticosteroids is not desired. Marked suppression of the effect of the 11-oxycorticosteroids may be accomplished by omitting the water load and by expressing the response as a ratio of sodium to potassium. Grundy, Simpson, Tait, and Woodford (1952) have used this approach effectively in detecting a small quantity of material with a desoxycorticosterone-like effect in the presence of much 11-oxy-corticosteroid in adrenal cortical extract. This method fails, however, in expressing the difference between a smaller desoxycorticosterone-like action and the effect of an 11-oxy-corticosteroid.

In the present method, Reichstein's compound S was found to have an action like desoxycorticosterone, but on a weight basis, compound S was at most 1/20th as effective as DOCA. Compound S and hydrocortisone in 100 μg. doses had large, opposite effects on sodium output in the present method. When Simpson and Tait (1952) tested compound S and hydrocortisone, the effects of the two compounds expressed as sodium to potassium ratios were quantitatively comparable. These observations indicate that selection of experimental conditions and expression of response are determining factors in the result obtained. Certain basic information on the qualitative effects of the material to be tested can be obtained by individual measurement of sodium, potassium, and water output.

SUMMARY

A method has been described for estimation of adrenal corticosteroids affecting sodium and potassium excretion. The method provides a qualitative distinction between DOCA-like sodium-retaining steroids, inert compounds, and hydrocortisone-like steroids which increase output of sodium. A known error variance has been determined both for estimates based on Na output and on K/Na output ratio. The relative importance of variables affecting estimates of both of these responses has been separated and compared by statistical analysis of variance. The improved precision of estimation of DOCA-like steroids by K/Na ratio calculation has been presented in comparison with calculation based on Na alone. Advantages and defects of both calculations have been discussed. Correction can be made for bias of estimation when rats must be discarded from an assay. Responses to different doses of DOCA, Reichstein's compound S, cortisone, hydrocortisone, corticosterone and 11-dehydro-corticosterone have been described.

The method is suitable for measurements on biological fluids and requires relatively small amounts of material.

Acknowledgments

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BIOGENESIS OF THE ESTROGENS: THE FAILURE OF CHOLESTEROL-4-C^{14} TO GIVE RISE TO ESTRONE IN THE PREGNANT MARE

R. D. H. HEARD AND V. J. O'DONNELL[1]

Department of Biochemistry, McGill University, Montreal, Candaa

INTRODUCTION

IT HAS been established (for reviews of evidence see Gallagher *et al.* (1954); Heard *et al.*, 1954) that cholesterol functions in the animal organism as a precursor of the non-aromatic steroid hormones. Bloch (1945) demonstrated the conversion of deuterio-cholesterol to pregnane-3α,20α-diol in the human during pregnancy, and concluded that progesterone was derived from this sterol. Gallagher *et al.* (1954) showed in the human the transformation (presumably through testosterone) of C^{14}-cholesterol to androsterone and etiocholanolone, while perfusion of isolated beef adrenal glands with cholesterol-3-C^{14} led to 17-hydroxy-corticosterone and corticosterone (Zaffaroni, Hechter and Pincus, 1951). Biogenesis to progesterone, certain androgens and certain adrenocorticoids may thus be considered well established, but no evidence has been forthcoming to suggest that the estrogens may arise from cholesterol. Such a transformation *in vivo* demands, in addition to the established oxidative fission of the side chain, aromatization of Ring A with concomitant removal of the angular methyl group between Rings A and B, a series of reactions which has not been demonstrated within the animal organism. To test the hypothesis, one pregnant mare was injected with 56.6 million counts per minute of cholesterol-4-C^{14}; the estrone subsequently isolated from the urine of the following five days proved to be inactive radiochemically. *Equus caballus* was chosen for the experiment because it is the only species other than the human which excretes estrogens in quantity sufficient to admit isolation in crystalline state.

EXPERIMENTAL AND RESULTS

Melting points were taken on the Fisher-Johns micro-block; the recorded values are corrected.

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Radioactivity was determined in the windowless flow-gas counter operating at 40–50% efficiency. Absorption within each sample was determined by the addition of a known number of C^{14} counts to the various extracts, and the calculated correction factor was applied in each case (method of Heard, Thompson and Yates; see Heard *et al.*, 1954).

Administration of cholesterol-4-C^{14} and collection of excreta.

The sterol[2] (56.6 million counts per minute in 409 mg.) in 60 ml. of 95% ethanol was administered intrajugularly in 4 equal portions over a period of 24 hours to a Percheron mare in the 8th month of gestation.

Urine was collected in a rubber container (half-section of the inner tube of an automobile tire) fitted to the vulva and held in position by an appropriate abdominal harness. The contents were emptied several times daily, acidified to pH *ca.* 3, and stored at 5° C. under a layer of toluene. Each 24-hour output (average 5.95 l.) for 5 days was subjected separately to acid hydrolysis (below). Feces (6.3 kg.) were collected on the third day. Two samples of saliva were obtained by swabbing 3 and 30 minutes after the second injection.

Fractionation of urine

The urine was processed by a modification of the method of Cohen and Marrian (1934). It was acidified to pH 1 with concentrated hydrochloric acid, covered with an equal volume of toluene and refluxed for 2 hours. On cooling, the toluene layer was separated and the aqueous phase was extracted with the same solvent (2×one-half volume). The combined toluene extracts were washed with water (5×one-tenth volume) and concentrated *in vacuo* on the steam bath to *ca.* 1 l. The toluene concentrate of each 24-hour collection was counted separately. In view of the low daily excretion of radioactivity, it was deemed advisable to combine the concentrates of days 1 to 5 inclusive before further processing. The pooled toluene soluble material was then partitioned into acidic, strong phenolic, weak phenolic, and neutral fractions by extraction with, respectively, solutions of 10% sodium carbonate (1×one-half volume) and 5% sodium carbonate (2×one-half volume), 0.1 N sodium hydroxide and N sodium hydroxide (in each case, 3×one-half volume). The acidified carbonate and alkali extracts were then extracted with ether (3×one-half volume in

[2] The tracer cholesterol was kindly prepared by Dr. W. W. Zorbach by reduction of cholestenone-4-C^{14} with sodium borohydride (method of Schwenk, Gut and Belisle, 1951; and of Belleau and Gallagher, 1951). We are much indebted to Dr. P. Ziegler for adequate supplies of cholestenone-4-C^{14} (prepared by the method of Heard and Ziegler, 1951).

each instance). The combined ether extracts of each fraction and the remaining toluene were washed with water (each 3×one-tenth volume) and taken to dryness *in vacuo* on the steam bath. The total residual aqueous urine was concentrated to *ca.* 15 l. Qualitatively. this fraction showed radioactivity (*ca.* 230,000 counts per minute). Quantitative data and radioassay of the various organic fractions are given in Table 1.

TABLE 1. QUANTITATIVE DATA AND RADIO-ASSAY OF URINARY FRACTIONS

Fraction	Weight (gm.)	Counts per minute
Acidic	170.25	0
Strong phenolic	15.429	0
Weak phenolic	3.615	0
Neutral	17.868	67,875

Isolation of estrone

Estrone excretion was measured by Kober assay as 99 mg. per l. of urine (day 1). The weak phenols (3.615 gm.) were separated into ketonic (1.5536 gm.) and non-ketonic (0.9886 gm.) fractions with Girard's Reagent T (1936). From the former, light oils were removed by high vacuum distillation to 150° C., and the residue was sublimed at 150°–175° C. Recrystallization of the sublimate (m.p. 221°–232° C.) from 95% ethanol gave crude estrone, m.p. 241°–243° C. which was further purified by elution with benzene from a column of Celite previously impregnated with 0.4 N sodium hydroxide solution. This chromatographic method (a modification of that of Haenni, Carol and Banes[3]) was developed in these laboratories by Dr. S. Solomon for the separation of estrone, equilin and equilenin, and will be published in full detail in a later communication. The estrone eluates were recrystallized from ethanol to give the final product, m.p. 262°–263° C. and m.p. 260°–262° C. on admixture with authentic estrone, m.p. 262°–264° C. Further identification is provided by the absorption spectrum in sulphuric acid (Fig. 1). It is noted that the "authentic" estrone shows a weak maximum at *ca.* 350 mμ. (where equilin exhibits strong absorption) which represents slight contamination with equilin; estrone purified through the Celite-NaOH chromatographic system is free of equilin. Plated at a concentration of 1 mg. per 5 cm.^{2}, the isolated estrone showed no radioactivity.

Isolation of benzoic acid

From the acidic fraction, benzoic acid was obtained directly by sublimation *in vacuo* at 60° C. and purified by repeated recrystallization from 95%

[3] We wish to thank Dr. J. Carol for details in advance of publication.

ethanol. The benzoic acid (m.p. and admixture m.p. 122°–123° C.) was not radioactive.

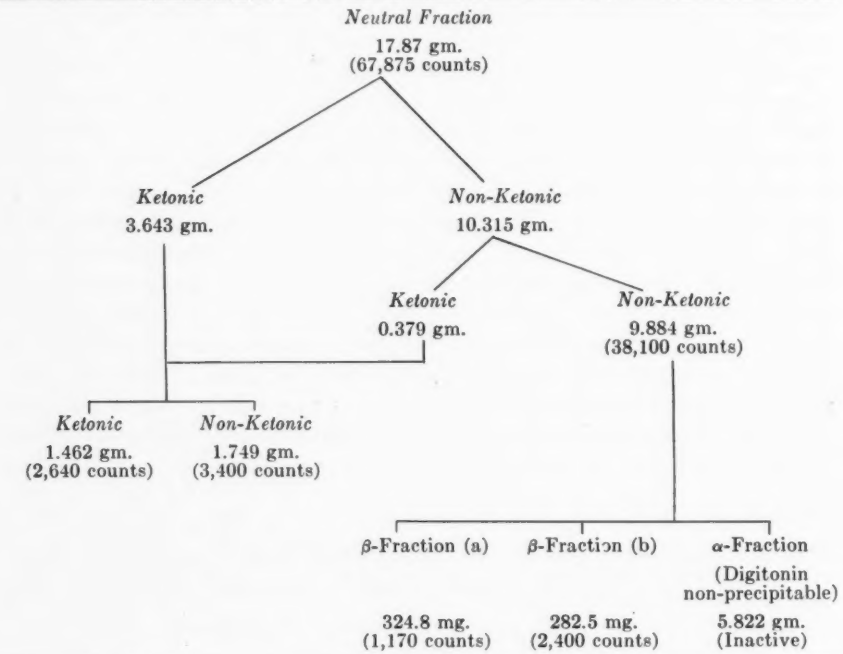
Fractionation of neutral material

The neutral toluene concentrates were evaporated to dryness and divided into ketonic and non-ketonic portions (Girard and Sandulesco, 1936). The non-ketones were further separated into digitonin-precipitable (β) and digitonin-non-precipitable (α) fractions (following the techniques described by Marker, Rohrmann and Wittle, 1938, and by Brooks, Klyne, Miller and Paterson, 1952). Two digitonides were obtained: (a) that precipitated in 95% ethanol (24 hours in the refrigerator), and (b) that obtained from the concentrated filtrate of (a) by precipitation with ether (20 volumes). Each was separately dissociated in pyridine, and the liberated β-alcohols were extracted with ether. Quantitative data and radio-assays are summarized in Table 2.

Feces

Feces (6.3 kg.) collected on day 3 were leached on a large Buchner funnel with chloroform (63 l.). The chloroform extract was distilled *in vacuo* on

TABLE 2. FRACTIONATION OF NEUTRAL MATERIAL



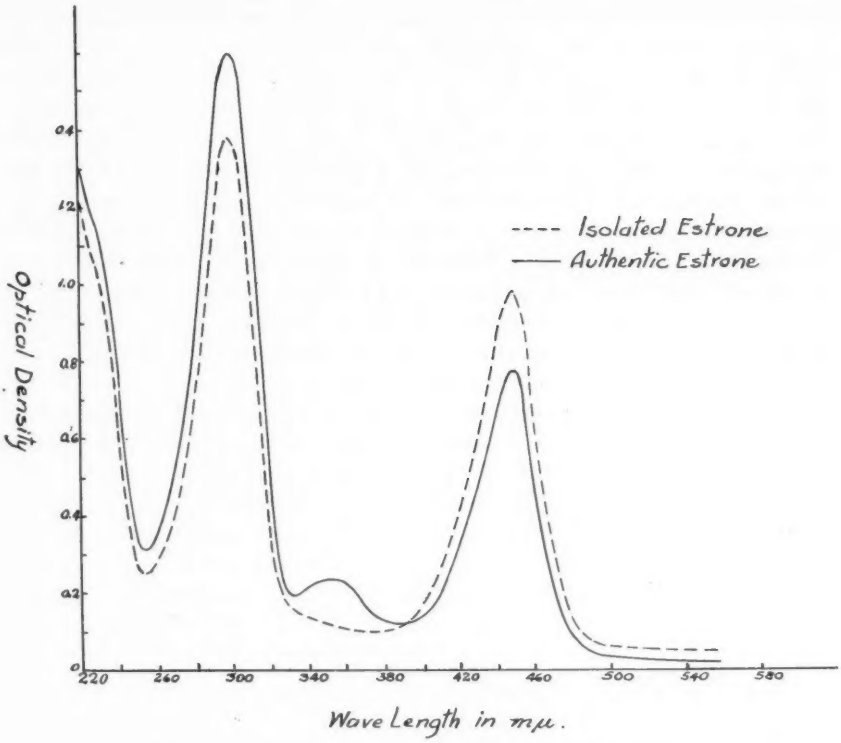


FIG. 1. Absorption spectrum in sulphuric acid of estrone.

the steam bath to 2520 ml. and an aliquot of this (0.06 ml. representing 166.6 mg. of feces) was plated. This counted 4 disintegrations per minute above background, which corresponds to a total of 189,000 counts per minute for the sample. Fecal excretion is therefore appreciable, but is not a high proportion of the total injected radioactivity.

Saliva

The absorbent cotton swabs taken 3 and 30 minutes after the second injection were extracted with methanol and aliquots of these two extracts were plated for radio-assay. Qualitatively, slight secretion of C^{14} was noted (respectively 250 and 400 counts per minute per swab).

DISCUSSION

From the data (Table 1) it is apparent that only the neutral excretory products contain appreciable radioactivity. Although the weak phenolic

estrogen fraction exhibited no detectable count, it was considered necessary to isolate estrone in chemically pure state, as a low C^{14} content in the latter might well be masked by a very high proportion of non-radioactive extraneous phenols. The zero count of the isolated estrone clearly indicates that it is not derived from cholesterol of the maternal circulation. The significance of this negative observation becomes apparent from the following calculations. The total body cholesterol of a 750 kg. mare is approximately 750 gm., about half of which is contained in the central nervous system where the rate of turnover is negligible. Waelsch, Sperry and Stoyanoff (1940) have demonstrated that cholesterol is synthesized slowly, if at all, in the brain of adult rats, and Bloch, Berg and Rittenberg (1943) showed the complete absence of deuterio-cholesterol from the brain and spinal cord of dogs 3 days following the last intravenous injection of the tracer sterol. Based on the assumption that the administered C^{14}-cholesterol is uniformly diluted with the remaining 375 or so gm. of body cholesterol before reaching the site of estrogen formation (an improbable occurrence), the count of the cholesterol would be 151, and that of estrone derived therefrom, 209. In that a count of 2 above background is readily measurable, it is evident that little or no estrone arises from this source.

Only a small proportion of the administered C^{14} is excreted in 5 days. Approximately 0.6% appears in the urine, and not more than *ca.* 3% in the feces (estimated from a single sample). Presumably much is stored as unchanged cholesterol, and much is degraded to bile acids. Storage capacity is marked in all tissues except brain and spinal cord, and conversion to cholic acid has been proven in the dog (Bloch, Berg and Rittenberg, 1943). Most of the urinary C^{14} was water soluble, but the appreciable activity of the neutral organic fraction warranted further separation (Table 2) into 4 fractions: (a) ketonic, (b) and (c) two non-ketonic β-alcoholic fractions (differentiated by solubilities of the digitonides), and (d) a digitonin non-precipitable non-ketonic portion. All but (d) retained radioactivity. Very marked loss of C^{14} accompanies this separation procedure, which can only be attributed to escape of volatile C^{14}-metabolites in the course of several evaporations to dryness *in vacuo*. In that most known neutral steroids of mares' pregnancy urine are pregnane or allopregnane derivatives, it is highly probable that cholesterol is converted, through progesterone, to these compounds; this has been demonstrated in the human by Bloch (1945). It is unlikely that any of the radioactivity of the β-alcoholic fraction is due to the excretion of cholesterol-4-C^{14}, as this sterol has never been encountered in systematic searches of the digitonin-precipitable neutral alcohols from huge volumes of mares' pregnancy urine (Marker and Rohrmann, 1938; Heard and McKay, 1939, and subsequent unpublished observations; Brooks, Klyne, Miller and Paterson, 1952).

SUMMARY

1. A pregnant mare was injected intrajugularly with cholesterol-4-C^{14} and the excreta were examined for C^{14}-labeled metabolic products. Radioactivity was excreted in urine, feces and saliva.

2. The steroid-containing portion in the urine extracts yielded estrone which was non-radioactive. The neutral ketonic and non-ketonic fractions contained considerable C^{14}.

3. It is thus concluded that in the pregnant mare cholesterol administered to the maternal circulation in this manner does not give rise to estrone, but very probably functions as a precursor of pregnane and allopregnane derivatives.

Acknowledgments

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REGULATION OF THE RAT THYROID IN SHORT-TERM IODINE DEFICIENCY[1]

N. S. HALMI

Department of Anatomy, State University of Iowa, Iowa City, Iowa

THE central role of iodine deficiency in the etiology of simple goiter has been firmly established (Marine, 1935). Thyroid hyperplasia readily develops in rats maintained on a goitrogen-free low iodine diet (Levine *et al.*, 1933, Remington and Remington, 1938). According to the most generally accepted theory of the pathogenesis of thyroid changes in iodine deficiency, the inadequate supply of iodine curtails the formation of thyroid hormone, and the resulting hypothyroid state leads to compensatory hypersecretion of pituitary thyrotrophin (TSH) (Marine, 1935; Greer, 1950; Riggs, 1952). In addition to thyroid enlargement, further signs of increased TSH production were described in rats fed low iodine diets, such as augmented thyroid cell height, pituitary changes similar to those seen in hypothyroid animals and enhanced thyroidal radioiodine collection (Leblond and Mann, 1942). Chapman (1941), however, reported that thyroid cell height increases in response to low iodine intake even in the absence of the pituitary.

Money *et al.* (1952), examining the temporal sequence of thyroid changes in rats on an iodine deficient (Remington) diet, found an increased uptake of I^{131} within a few days after the regimen had been started, whereas thyroid enlargement did not become manifest until over 3 months later. They pointed out that the uptake of a greater percentage of an I^{131} label by the thyroid in short-term iodine deficiency does not necessarily indicate an increased collection of I^{127}, but may very well be due to the labelling of a diminished pool of iodide by the isotope, without any change in thyroid function. Maloof *et al.* (1952), on the other hand, reported an increase in both thyroid size and I^{131} uptake in rats which had been fed a Remington diet for 11 days.[2]

Since basic uncertainties exist concerning the mechanism as well as the

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[2] The discrepancies between the results of these two groups of workers are particularly hard to reconcile since the diet used by Money *et al.* was stated to contain 45 μg. of iodine/kg. and that fed by Maloof *et al.* 90 μg./kg.

time of onset of the thyroid's reaction to low iodine intake, an analysis of pituitary-thyroid interplay in iodine deficiency of short duration appeared indicated. Groups of intact, hypophysectomized and hypophysectomized-TSH treated rats were fed a Remington diet, while their respective controls were maintained on a high iodine regimen. In addition to thyroid weight and mean cell height determinations, thyroid:serum iodide gradients (T/S) were examined in all groups, using tracer doses of I^{131}. The latter measurement is eminently useful, since a) its magnitude is a very sensitive index of the level of thyrotrophic stimulation (VanderLaan and Greer, 1950; Ingbar and Roberts, 1952; Halmi *et al.*, 1953), and b) the T/S for I^{131} can be regarded to be identical with that for I^{127}, its alterations thus always being indicative of changes in thyroid function. In some intact animals the effect of dietary iodine intake on the thyroid was tested by administering large doses of I^{131}-labelled stable iodide, since recent studies (Halmi, 1954) have shown that TSH expands the capacity of the thyroidal iodide "trap," which is approximately 20 mg. of iodide per 100 gm. thyroid tissue in normal rats. Finally, the pituitaries of iodine deficient rats were examined for histological signs of TSH hypersecretion.

MATERIALS AND METHODS

All rats used in this study were young adult males of the Sprague-Dawley strain. Experiments were carried out on 5 groups of animals.

Iodine deficiency was produced by feeding a Remington diet[3] for the last 19 or 20 days before necropsy. Controls were fed Rockland pellets[4] for the same length of time and sacrificed simultaneously with the corresponding experimental groups. All rats received tap water.

In Experiment I intact rats (final weight 200–250 gm.) were used. Experiments II, III and IV were carried out on hypophysectomized animals (120–180 gm.) obtained from a commercial source.[5] In Experiments II and IV the rats were started on the described dietary regimens 9 days after hypophysectomy, in Experiment III one day after operation. The animals in Experiment IV received daily subcutaneous injections of .05 USP units of TSH (=1 mg. USP Thyrotropin Reference Substance) for the last 5 days before autopsy. At the end of the experimental period all rats of Experiments I–IV were given a tracer dose (2–10 μc.) of carrier-free radioiodide subcutaneously, 45 minutes after a single injection of 6 mg. of propylthiouracil, and sacrificed 1 to $1\frac{1}{2}$ hrs. later. The thyroids were weighed on a Roller-Smith torsion balance. Thyroid: serum

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[3] "Low Iodine Test Diet," Nutritional Biochemicals Corporation, Cleveland. Composition: Yellow corn meal (grown in iodine deficient area) 78%, wheat gluten 18%, brewer's yeast, U.S.P., 2%, calcium carbonate 1% and sodium chloride 1%. No statement as to the iodine content of the diet was available. Reported values for similar regimens range from 15 μg. of iodine/kg. (Levine *et al.*, 1933) to 90 μg./kg. (Maloof *et al.*, 1952).

[4] Stated iodine content 12 mg./kg.

[5] Hormone Assay Laboratories, Chicago.

iodide concentration ratio (T/S) determinations were performed as described in a previous publication (Halmi *et al.* 1953). One lobe of each thyroid was fixed in SUSA, embedded in paraffin, cut at 5 μ and stained with Gomori's trichrome. Mean cell heights were determined in projected representative sections by measuring one cell per follicle in 20 follicles picked at random from each gland. The pituitaries were prepared for histological examination according to the technique of Halmi (1952a).

In Experiment V intact rats (final weight 250–300 gm.) received 20 mg. of I^{131}-labelled NaI^{127} of known specific activity in lieu of the carrier-free tracer 1 to $1\frac{1}{2}$ hrs. before autopsy. Thyroid and serum iodide concentrations (T and S) were calculated, disregarding the iodide that had been present prior to the injection, and the concentration of "trapped" thyroidal iodide expressed as T minus S/2.5 (Halmi, 1954).

RESULTS

The results of Experiment I are shown in Figure 1. It can be seen that low iodine intake, under the conditions of the experiment, had no morphological stimulating effect on the thyroids of intact rats. In fact, mean acinar cell height was greater ($p = 0.02$) in the Rockland diet fed controls. In accordance with this latter observation, the pituitaries of the rats maintained on the high iodine diet showed histological signs (low number of fully granulated thyrotrophs, many thyroidectomy cells exhibiting incipient vacuolation) suggestive of a higher TSH output than that of the iodine deficient animals, whose hypophyses contained numerous heavily granulated thyrotrophs and but few thyroidectomy cells.[6] Nevertheless, the thyroid:serum iodide concentration ratio (T/S) for the Rockland group was close to the "normal" value of 25 (VanderLaan and VanderLaan, 1947; Halmi *et al.*, 1953), whereas that of the low iodine group was greatly elevated and well within the range of the T/S values found in rats with thiouracil or propylthiouracil induced goiter (VanderLaan and VanderLaan, 1947; Greer, 1952; Wyngaarden *et al.*, 1953; Bogdanove and Halmi, 1953; Halmi, 1954).

The findings obtained in Experiment V are summarized in Table 1. The administered dose of 20 mg. of NaI^{127} was apparently sufficiently high to saturate the thyroidal iodide "trap" of the controls, since the concentration of "trapped" iodide in their thyroids was of the order of magnitude of the normal gland's capacity level (Halmi, 1954). Reduced dietary iodine intake markedly expanded the capacity of the thyroidal iodide "trapping" mechanism.

Experiments II and III (Fig. 2) were performed in order to examine the influence of dietary iodine levels on the thyroid of hypophysectomized rats. In neither experiment was there a morphological difference between the thyroids of the low and high iodine groups, and the iodine deficient diet

[6] The histophysiology of TSH secretion has been discussed in an earlier note (Halmi, 1952b).

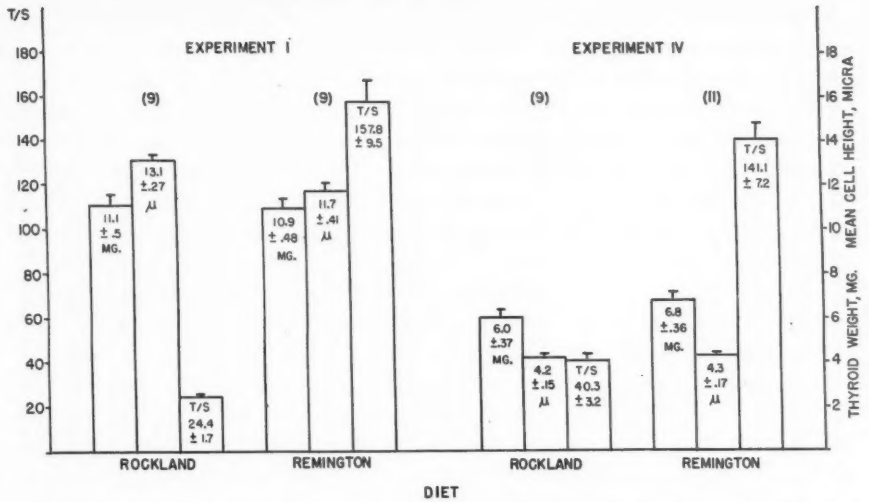

FIG. 1. Effect of dietary iodine intake on thyroid weights, mean acinar cell heights and thyroid:serum iodide gradients (T/S) in intact rats (Experiment I) and hypophysectomized rats injected with TSH (Experiment IV). Figures in parentheses represent number of rats per group; vertical bars mean values; vertical lines the positive segment of the standard error.

failed to prevent the atrophy of the follicular cells even when it was started 24 hrs. after ablation of the pituitary (Experiment III). On the other hand, the T/S values were significantly ($p < 0.01$) higher in both Remington diet fed groups than in the respective controls maintained on the Rockland regimen.

Experiment IV (Fig. 1) demonstrated that low iodine intake can greatly enhance the response of the T/S of hypophysectomized rats to exogenous TSH without concomitant morphological evidence of greater thyroid activation.

TABLE 1. THYROID: SERUM IODIDE CONCENTRATION (T/S) RATIOS, THYROID AND SERUM IODIDE CONCENTRATIONS AND ESTIMATED THYROIDAL "TRAPPED" IODIDE CONCENTRATIONS IN INTACT RATS 1 TO 1½ HOURS AFTER THE SUBCUTANEOUS INJECTION OF 20 MG. OF I^{131} LABELLED NaI^{127}

Diet	No. rats	T/S ratio	*T* Mg. of I^{127} per 100 gm. thyroid	*S* Mg. of I^{127} per 100 ml. serum	*T minus S*/2.5 Mg. of "trapped" I^{127} per 100 gm. thyroid
Rockland	11	2.4 ± .1*	30.2 ± .8	13.1 ± .7	24.9 ± .9
Remington	9	4.3 ± .3	70.4 ± 3.7	16.4 ± .5	64.1 ± 3.4

* Mean ± standard error.

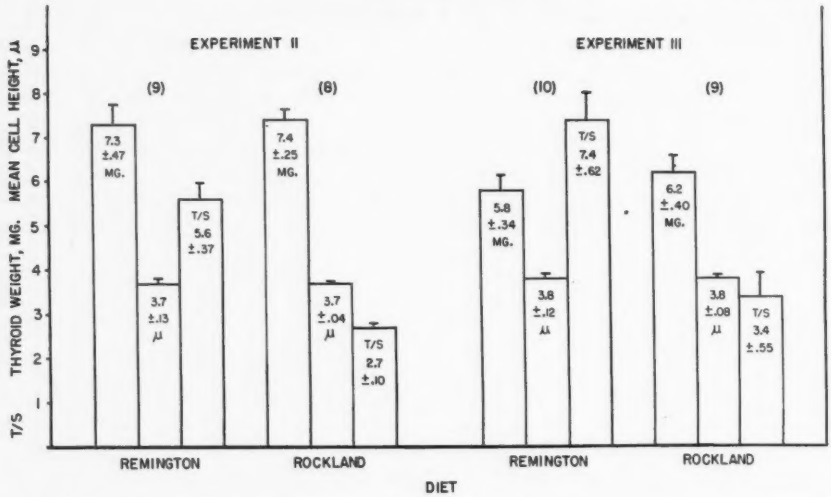

FIG. 2. Effect of dietary iodine intake on thyroid weights, mean acinar cell heights and thyroid-serum iodide gradients (T/S) in hypophysectomized rats sacrificed 29 days (Experiment II) or 20 days (Experiment III) postoperatively. Figures in parentheses represent number of rats per group; vertical bars mean values; vertical lines the positive segment of the standard error.

DISCUSSION

Although the thyroids of the low iodine diet fed intact rats in this study failed to exhibit any morphological signs of excessive thyrotrophic stimulation, the elevation of the thyroid:serum iodide gradient (T/S) and the increased capacity of the thyroidal iodide "trap" showed that *the iodide concentrating mechanism of these thyroids was greatly activated.* In view of the histological appearance of the pituitary enhanced release of TSH was considered unlikely. Thus the possibility had to be examined that iodine deficiency directly stimulates the thyroid (Chapman, 1941, Astwood, 1949).

The Remington diet fed hypophysectomized rats of Experiments II and III showed T/S values which were much lower than those of normal rats, but significantly higher than those of the hypophysectomized animals maintained on Rockland chow. This suggests that the high T/S of the iodine deficient rats in Experiment I cannot be ascribed solely to a direct activation of the thyroidal iodide concentration mechanism by iodine deficiency, but that the level of iodine intake does exert an influence on the iodide "trap" even in the absence of TSH.[7]

[7] The magnitude of the difference between the T/S values of the low and high iodine groups in Experiments II and III indicates, in the light of an earlier study (Halmi, 1954), that it cannot have been merely due to a partial saturation of the iodide "trap" in the Rockland-diet-fed animals.

Further work will have to clarify how graded dietary iodine levels affect the T/S of intact rats. The T/S of the Rockland controls in Experiment I was the same as that of rats fed Purina Chow (VanderLaan and VanderLaan, 1947), although the iodine content of the latter feed is considerably lower.[8] However, the results obtained by feeding commercial chows are difficult to analyze because of the possibility that these diets may contain positive goitrogens, as suggested by the great thyroid cell height and the histological picture of the pituitary in the intact controls of this study.

Experiment IV afforded an explanation for the diochotomy between the morphological picture and the iodide collecting activity of the thyroid in the iodine deficient intact rats of Experiment I. It demonstrated that *low iodine intake can potentiate the effectiveness of exogenous TSH on the T/S without increasing the influence of the hormone on thyroid size and cell height.* The inference appears justified that low dietary iodine levels may modify the action of endogenous TSH in a similar fashion. *In the course of adaptation to iodine deficiency more efficient utilization of serum iodide by the thyroid thus may precede both histological activation of the gland and increased output of TSH,* as exemplified by the Remington diet fed rats in Experiment I. It is also possible that the first signs of morphological stimulation of the thyroid in turn antedate the increased discharge of TSH from the pituitary, which probably represents a reaction to the drop in the level of circulating thyroid hormone. Maloof *et al.* (1952) found that a dose of exogenous TSH, which was ineffective when given to normal rats on Purina Chow, produced marked thyroid enlargement when administered to animals on a Remington regimen.

The described potentiation of the T/S response to TSH by low iodine intake seems to explain some paradoxical observations previously made in this laboratory (unpublished experiments performed in collaboration with E. M. Bogdanove, B. N. Spirtos and H. J. Lipner). We found that the T/S of rats starved for 5 days was either normal or elevated, whereas thyroid cell height was invariably markedly reduced. D'Angelo (1951) reported that the blood TSH levels of starved rats are significantly diminished. It seems logical to conclude that our starved animals, which must have been in a state of negative iodine balance, secreted decreased amounts of TSH, as indicated by the morphological regression of the thyroid, while the sensitivity of their thyroidal iodide collecting mechanism to TSH was augmented because of the iodine deficiency.

It may not be fortuitous that low iodine intake and propylthiouracil (PTU) (Halmi *et al.*, 1953) both enhance the T/S response of hypophysectomized rats to TSH. It has been suggested (Rawson and Money, 1949)

[8] Catz *et al.* (1953): 937 μg. of iodine/kg; Maloof *et al.* (1952): 3.2 mg./kg.

that iodine is the physiological inactivator of TSH in the thyroid. Wright and Trikojus (1946) considered the possibility that nascent elemental iodine may combine with TSH within the thyroid, since they and Albert *et al.* (1946) demonstrated that free iodine inactivated TSH containing pituitary extracts *in vitro*. In the case of iodine deficiency the decreased amounts of iodine in the thyroid may be used primarily in the formation of thyroid hormone and the inactivation of TSH may suffer. PTU, on the other hand, is believed to react with nascent elemental iodine within the thyroid, and thus also may prevent *in vivo* iodination of TSH.[9] Even if it be assumed that both iodine deficiency and PTU treatment interfere with the inactivation of TSH by iodine, thus rendering any given amount of the hormone more effective, it has to be emphasized that, under the conditions of our experiments, they greatly enhanced the reaction of the thyroidal iodide "trapping" mechanism to TSH but had little or no augmenting influence on the morphological response of the gland. If this were simply due to the greater inherent sensitivity of the iodide "trap" to TSH, the combination of histologically inactive thyroids and T/S values over 100 could be duplicated by giving appropriate doses of TSH to hypophysectomized rats receiving no goitrogen and adequate amounts of iodine. However, so far we have been unable to elevate the T/S beyond 90 by administering large doses of TSH alone to hypophysectomized rats fed a supplemented Rockland regimen (Halmi *et al.*, 1953), whereas even comparatively small amounts of TSH raised the gradient to 130–150 in PTU treated or iodine deficient animals. Furthermore, as mentioned above, an increase of the T/S was observed in several groups of starved rats whose thyroid morphology suggested a decline in the output of TSH. It thus appears necessary to postulate that *both iodine deficiency and PTU treatment*, in addition to other possible influences on the TSH molecule and/or its inactivation by the thyroid, *either sensitize the iodide concentrating mechanism to TSH or specifically potentiate the action of the trophic hormone on the thyroidal iodide "trap."* This effect may be mediated through some change in thyroidal iodine metabolism common to both conditions.

SUMMARY

1. Thyroidal weights, mean acinar cell heights and the performance of the thyroidal iodide concentrating mechanism were examined in groups of intact, hypophysectomized and hypophysectomized-thyrotrophin (TSH) treated rats maintained on a low iodine (Remington) diet or a high iodine (Rockland) regimen for 19–20 days.

[9] Goitrogens have also been reported to reactivate iodinated TSH and to increase the biological activity of untreated TSH-containing pituitary extracts (Albert *et al.*, 1947).

2. Although the thyroids and the pituitaries of intact iodine deficient rats showed no morphological evidence of thyrotrophic activation, the thyroid:serum iodide gradients (T/S) were greatly elevated and the capacity of the thyroids for "trapped" iodide was expanded.

3. The low iodine regimen had no effect on the morphological regression of the thyroids of hypophysectomized rats and the T/S in these animals was also lower than in normal rats, albeit significantly higher than in Rockland diet fed hypophysectomized controls. An effect of iodine intake on the thyroidal iodide "trap" in the absence of TSH is suggested.

4. Iodine deficiency markedly increased the effectiveness of exogenous TSH on the T/S of hypophysectomized rats, without enhancing the influence of the hormone on thyroid morphology. A comparison is made with previously encountered similar effects of propylthiouracil treatment and the mechanisms possibly involved are discussed.

5. It is concluded that an improvement in the efficiency of thyroidal iodine uptake may precede other changes of the pituitary-thyroid axis in iodine deficient states, owing to an augmented response of the thyroidal iodide "trap" to TSH.

Acknowledgment

I wish to thank B. N. Spirtos for his invaluable aid in carrying out these experiments.

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NOTES AND COMMENTS

EFFECTS OF TOTAL BODY X-IRRADIATION ON THE RAT PITUITARY

In 1952 Botkin, Praytor, Austing and Jensen reported on the response of the thyroid gland to total body x-irradiation. Using male albino rats subjected to 1000 r of body x-irradiation they found a transitory increase in activity followed by a continuous fall in activity starting about 24 hours post irradiation. Patt, Swift, Tyree, and John (1947), on the other hand, under similar conditions of irradiation found an increase in adrenal gland weight and a loss of cholesterol in that organ. Botkin and co-authors correlated these results and suggested that under the stress of x-irradiation there is an initial increase of TSH and ACTH, subsequent to which there is a heightened elaboration of ACTH at the expense of the TSH. It was further intimated that the systemic responses might be mediated through the pituitary. Similarly Mateyko, Edelmann, Charipper and Gordon (1951) reported that total body x-irradiation caused an acceleration of adrenocorticotrophin synthesis, first at the expense of gonadotrophins and after 24 hours, of thyrotrophin.

The purpose of this paper is to report on the histopathological changes in the pituitaries of the rats used by Botkin and co-workers.

Following irradiation, the pituitaries were fixed in Bouin's solution and after routine dehydration were stained with Gomori's aldehyde fuchsin stain (Gomori, 1950; Halmi,

TABLE 1. MEAN DIFFERENTIAL COUNTS OF THE PITUITARY CELLS OF NORMAL RATS AND THOSE EXPOSED TO TOTAL BODY X-IRRADIATION

Days post irradiation	Differential counts (%)			
	Chromophobes	Acidophiles	Beta cells	Delta cells
0 (controls)	52.7	39.3	5.3	2.7
1	40	58	0.75	1.2
2	37	60	0	3
3	38.5	59	0	2.5
4	35.5	62.5	0	2
5	35.5	63	0	1.5

1950). This stain delineates the cell types of the pituitary by differentially staining the chromophobes, acidophiles, and two classes of basophiles—the so-called beta cells or "Gomori-positive" cells, and the delta cells, which have a reniform nucleus and a clear cytoplasm. A series of normal rat pituitaries was used for control purposes.

The pituitaries from the normal and irradiated rats were examined histologically and differential cell counts were made. The experimental intervals used were 1, 2, 3, 4, and 5 days after exposure. Six glands were used for each time period and several thousand cells from representative sections were counted. The results are seen in Table 1, where the means for each time interval are presented.

At 24 hours post irradiation there is a virtual absence of basophilic granules (beta

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cells) in the pituitaries. The same sections show a marked increase in acidophiles from 39.3% to approximately 60%. The chromophobes are diminished by an almost equal amount. After 24 hours, the "Gomori-positive" granules are completely gone, while the elevated acidophile count persists.

These findings correlate very closely with the biochemical data on thyroid function (Botkin *et al.*, 1952) and adrenal function (Patt *et al.*, 1947), and support the thesis that the beta cells are the source of TSH and the acidophiles the source of ACTH. (See also Purves and Griesbach, 1951 and Finerty and Briseno-Castrejon, 1949.) The continued high acidophile count suggests that under a certain extreme stress ACTH production may become paramount and assume precedence over other hormonal secretions from the hypophysis.

An additional finding after irradiation is a steady decrease in the wet weights of the pituitaries, accompanied by an increased total cell count per unit area of the glands (Table 2). The cells themselves show a slight but perceptible shrinkage. Most likely this

TABLE 2. MEAN WET WEIGHTS AND TOTAL CELL COUNTS PER HIGH POWER FIELD IN PITUITARY GLANDS FOLLOWING IRRADIATION

Days post irradiation	Weight in mgms.	Total cells per field
0 (controls)		141
1	6.53	158
2	5.52	191
3	5.55	185
4	5.03	203
5	4.30	229

is a manifestation of intracellular water loss. A plot of the weights and cell counts has a linear form which indicates a constant rate of change. The effect is not specific for the pituitary, however, since the total body weight decreases in a parallel fashion.

ROY KORSON and A. L. BOTKIN

Department of Biochemistry
Army Medical Research Laboratory
Fort Knox, Kentucky

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WEIGHT RESPONSE OF IRRADIATED ACCESSORY SEX ORGANS OF THE CASTRATE MALE RAT TO TESTOSTERONE PROPIONATE

The accessory sex organs (prostate, coagulating gland and seminal vesicles) of the male rat castrated at puberty show practically no spontaneous growth. However they maintain the potentiality for growth for a long time after castration. The growth response of the accessories to testosterone propionate (TP) is the same as early as one week and as late as fifty weeks after castration (Hays and Mathieson, 1945). Response to TP is associated with profuse mitotic activity and rapid formation of desoxyribonucleic acid (DNA) as indicated by tracer studies using P^{32} (Fleischmann and Fleischmann, 1952).

It has been claimed that irradiation with x-rays can interfere with the formation of DNA and that this interference constitutes the mechanism or part of the mechanism of the action of x-rays on growing tissues (Hevesy, 1945; Holmes, 1947). It occurred to us that the growth of the accessory sex organs of the castrate male induced by administration of TP might be used for a study of these problems. As a preliminary step we decided to study the weight response of irradiated accessories of the castrate rat to treatment with TP.

MATERIAL AND METHODS

Male rats of the Wistar strain were castrated at the age of 30 days and were used in groups of 6 or 12 from 1 to 3 months after castration. X-ray was given under amytal sodium anesthesia. The animal was shielded with lead (2 mm. thick). Irradiation was given through a portal centered over the pelvis. The diameter of this portal was 2.4 cm. 10,000 r were given in 8 minutes 10 seconds at 12 cm. distance with inherent filter (8 ma, 130 KV). TP was given by subcutaneous injection of 1 mg. of TP dissolved in 0.2 ml. of peanut oil (Perandren Ciba). TP was given immediately after irradiation to rats of Group D; the rats were sacrificed 72 hours after administration of TP. The following control groups were set up: Untreated castrate rats (Group A), rats killed 72 hours after irradiation (Group B) and rats killed 72 hours after administration of TP (Group C). In a second experiment rats were irradiated 24 hours after administration of TP (Group F) and sacrificed 48 hours after irradiation. The control group (Group E) in this experiment consisted of rats which had been given TP 24 hours before termination of the experiment. At the termination of each experiment the accessories were removed under deep anesthesia with amytal sodium and weighed to the nearest mg. The results are summarized in Table 1 together with a statistical evaluation.

TABLE 1. WEIGHT OF ACCESSORY SEX ORGANS AFTER TREATMENT WITH X-RAY AND TP

Group	No. of rats	After x-rays, hours	After TP, hours	Accessories	
				Average, mg.	S.D., mg.
A	6	—	—	52.8	4.6
B	6	72	—	49.0	10.2
C	6	—	72	109.7	8.6
D	12	72½	72	84.4	9.2
E	6	—	24	63.7	4.3
F	12	48	72	88.7	8.8

Statistical comparison of weights:

Groups compared	P
A–B	0.43
A–C	<0.01
A–D	<0.01
C–D	<0.01
E–F	<0.01

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DISCUSSION

Irradiation in the dose given has no or little effect on the weights of the accessories of the castrate male rat as indicated by comparison between Groups A and B. The well known effect of TP is evidenced by comparison of Groups A and C. This response to TP takes place even after irradiation with doses of x-ray as high as 10,000 r. This becomes evident by the difference between the weights of Group A and Group D. However the growth response is less in the rats, which had received both TP and x-ray (Group D) than in the rats which had received TP only (Group C). It is of interest that the accessories of rats, which had been stimulated with TP for 24 hours continue to grow during the next 48 hours despite irradiation with 10,000 r. This is evidenced by the difference between the weights of Group E and Group F. Further studies employing cytological and cytochemical methods are necessary to determine the nature of the combined effects of irradiation and hormonal stimulation; these studies are being undertaken.

SUMMARY

Under the conditions of the experiments outlined above the growth induced by testosterone propionate in the accessory sex organs of castrate male rats is only partially inhibited by irradiation with x-rays. This preparation offers a good opportunity for the study of the effect of x-rays on growth of tissues due to a specific stimulation of growth.

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WALTER FLEISCHMANN and JOHN T. BRACKIN, JR.
Veterans Administration Hospital
Oteen, North Carolina, and
Abington Memorial Hospital
Abington, Pennsylvania

PROGESTATIONAL ACTIVITY OF ORALLY ADMINISTERED 17α-ETHINYL-19-NORTESTOSTERONE

The need for orally active progestational compounds has resulted in the testing of many substances. A potent oral progestogen, 17α-ethinyl testosterone (pregneninolone) was described by Inhoffen and Holweg (1938). Oral administration of 4 mg. of this compound to estrogen-primed, infantile rabbits resulted in positive progestational proliferation. Under the same experimental conditions, a 60 mg. oral dose of progesterone was ineffective. Zondek and Rozin (1939) reported on the induction of uterine bleeding in

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TABLE 1. PROGESTATIONAL ACTIVITY OF ORALLY ADMINISTERED 17α-ETHINYL-19-NORTESTOSTERONE USING THE CLAUBERG TEST

Total dose (mg.)	Progestational proliferation	
	17α-ethinyl-19-nortestosterone	17α-ethinyl testosterone
5.0	—	4,3,3,3,3*
4.0	4,4,4,4,3,3,3,3,2,2	2,2,0
3.0	4,4,3	2,2,2,1
2.0	3,3,3	2,2,2
1.0	4,3,3,3,3,2	2,2,2,2,1,1
0.5	3,3,2,2,2,1,1,1	2,1,1,1
0.3	3,3,2,2,2,2,2,1,1,1,1	0,0,0,0
0.2	2,2,2,2,1,1,1,1,1,1,0,0,0	—
0.1	2,1,1,1,1	—
0.05	1,0,0,0,0	—
0.03	0,0,0,0,0,0	—

* Each number represents degree of progestational proliferation for one animal.

women by the oral administration of pregneninolone. They concluded that the effective dose by mouth is about 6 times greater than the effective intramuscular dose of progesterone.

In the course of a survey of orally active progestational steroids, we have compared the physiological activity of 17α-ethinyl-19-nortestosterone[1] with that of 17α-ethinyl testosterone (pregneninolone) according to the method of Clauberg (1930).

MATERIALS AND METHODS

Female New Zealand white rabbits, weighing 600–800 gm., received daily subcutaneous injections of 10 μg. estradiol-17β in 0.25 ml. corn oil for 6 days. Beginning on the seventh day, the test compound was administered by stomach tube in 1.0 ml. propylene glycol daily, for 5 days. Twenty-four hours after the last dose, the animals were killed. Body and uterine weights were recorded. Uteri were fixed in 4% neutral formalin, sectioned at 8 microns and stained with hematoxylin and eosin. The standard scale of McPhail (1934) was used in assessing the degree of progestational proliferation of the endometrium.

RESULTS AND DISCUSSION

The results of a series of bioassays of the two compounds are shown in Table 1. It can be seen that a total dose of 0.3 mg. pregneninolone (17α-ethinyl testosterone) is inactive whereas 0.5 mg. gives a progestational response comparable to that of 0.1 mg. 17α-ethinyl-19-nortestosterone. In addition, progestational proliferation produced by a 5 mg. dose of pregneninolone is comparable to that of 1 mg. 17α-ethinyl-19-nortestosterone. On the basis of this test therefore, the oral activity of the nor compound is at least 5 times greater than that of pregneninolone. However, the inherent quantitative variability of the response precludes a more precise estimate of physiological potency.

We have previously reported the high luteoid activity of parenterally administered 19-norprogesterone (Tullner and Hertz, 1953). Here, again we have found increased biological potency associated with the replacement of a methyl group at carbon 10 with hydrogen.

A compound with enhanced oral progestational activity may prove to be of experimental and clinical value.

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[1] We are indebted to Chemical Specialties Co., Inc., and Syntex, S.A. for the steroid compounds used in this investigation.

SUMMARY

Bioassay of 17α-ethinyl-19-nortestosterone, using the Clauberg method, demonstrates an oral activity approximately 5 times greater than that of 17α-ethinyl testosterone. This is a further instance of increased biological activity of a steroid when the methyl group at carbon 10 is replaced with hydrogen.

ROY HERTZ, WILLIAM TULLNER AND EDWARD RAFFELT

National Cancer Institute
National Institutes of Health
Public Health Service
Department of Health, Education, and Welfare
Bethesda, Maryland

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EFFECTS OF TRIIODOTHYRONINE ON TADPOLES[1]

Previous studies of triiodothyronine have shown this compound to have thyroid hormone-like activity, evidenced by its ability to prevent myxedema in humans (Gross *et al.*, 1952), and to possess five times the activity of thyroxine in preventing goiter formation in thiouracil-treated rats (Gross and Pitt-Rivers, 1953). It seemed of interest to investigate the ability of triiodothyronine to hasten amphibian metamorphosis and to estimate its potency in terms of thyroxine.

MATERIALS AND METHODS

Rana clamitans larvae were reared by the method suggested by Steinmetz (1950). Tadpoles of approximately 25 mm. were measured individually in a petri dish placed over graph paper ruled 20 lines per inch; they were assigned to five bowls so that each bowl contained 16 tadpoles of the same average length. All measurements are reported as 20ths of an inch. The bowls employed were 120 mm. crystallizing dishes containing 500 cc. of filtered[2] tap water (controls) or 500 cc. of filtered tap water plus one of the following compounds: 40 μg. thyroxine[3]; 10 μg. thyroxine; 10 μg. triiodothyronine[4]; or 2.5 μg. triiodothyronine. The bowls were aerated continuously, the water plus necessary compounds was changed every other day, and commercial baby food[5] was added 24 hours before the change of solution. Each time fresh solution was added each tadpole was measured and as animals died their data were discarded.

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[1] This research was done under the sponsorship of the Atomic Energy Commission.

[2] Fulflo-filter, BRIO 3/4, Commercial Filters Corporation, Boston.

[3] D-L Thyroxine, Squibb, E. R. Squibb & Sons, New York. 0.58 mg. dissolved in 2 cc. of dilute NaOH plus distilled water to make 10 μg. per cc., this solution added to bowl to give desired concentration.

[4] D-L, 3,5,3′Triiodothyronine, lot Pl-1871-A, generously contributed by Dr. E. L. Sevringhaus, Hoffmann-La Roche, Inc., Nutley, New Jersey. 0.44 mg. dissolved in 2 cc. of dilute NaOH plus distilled water to make 10 μg. per cc., this solution added to bowl to give desired concentration.

[5] Oatmeal, Gerber Products Co., Fremont, Michigan.

TABLE 1. LENGTHS OF TADPOLES DURING COURSE OF EXPERIMENT IN TWENTIETHS OF AN INCH REPORTED BY TREATMENT RECEIVED. FIGURES IN BRACKETS REFER TO STANDARD DEVIATION. NOT LESS THAN 10 ANIMALS IN EACH GROUP

Treatment, μg. per 500 cc.	Average tadpole length		
	Initial	10 days after beginning expt.	20 days after beginning expt.
None	15 (2)	17 (1)	19 (2)
2.5 Triiodothyronine	16 (2)	15 (1)	12 (1)
10 Thyroxine	15 (2)	15 (1)	14 (2)
10 Triiodothyronine	17 (4)	10 (2)	
40 Thyroxine	16 (4)	9 (1)	

The tadpoles were maintained on this regimen for ten days but in the case of the controls and those given the lower dose of each compound the experiment was allowed to run an additional ten days although no additional compound was added. Only data from the measurements made the first day and ten days later are reported for tadpoles given the higher doses of the two compounds; for an additional ten days in the case of the other three groups.

RESULTS

Ten days' immersion in either 40 μg. thyroxine or 10 μg. triiodothyronine per 500 cc. caused a decrease in tadpole body length. No marked decrease in body length of the tadpoles that received the lower dose of these compounds was apparent until the twentieth day.

DISCUSSION

The shortening of tadpole over-all length which followed the ten day immersion in either 40 μg. of thyroxine or 10 μg. of triiodothyronine per 500 cc. is considered to be

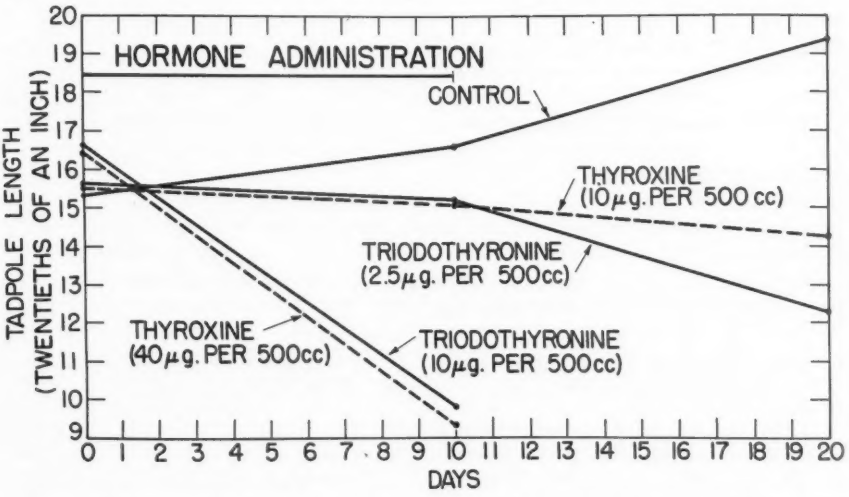

FIG. 1. Average of tadpole lengths during course of experiment plotted on ordinate in twentieths of an inch. Days of experiment plotted on abscissa. Hormone treatment (tadpoles immersed in concentration noted on each line) only during first 10 days of experiment. Each point represents no less than 10 animals.

evidence that both of these compounds hasten metamorphosis. Since both the compounds at this dose level were toxic, the number of tadpoles per compound was reduced to ten, and the experiment was effectively terminated at this point. The two lower dose groups (without further addition of the compounds) were measured for an additional 10 days and they also showed a marked shortening of over-all length.

An estimation was made of relative potency of triiodothyronine in terms of thyroxine on a molar basis. The criterion was the decrease in length of each tadpole after 10 days' immersion in the two compounds and the data were handled by an analysis of covariance (Snedecor, 1948) and the method of Bliss (1952). The relative potency calculated statistically showed that triiodothyronine was 3.8 times (3.3–4.4 at P=95%) more potent than thyroxine in hastening amphibian metamorphosis. The present finding that triiodothyronine exhibits maturation effects in amphibians adds merit to the suggestion of Gross and Pitt-Rivers (1953) that this compound must receive further consideration in the question of what is the thyroid hormone, not only in mammals but in the lower vertebrates as well.

SUMMARY

D-L triiodothyronine (2.5 or 10 μg. per 500 cc.) and D-L thyroxine (10 μg. or 40 μg. per 500 cc.) were effective in shortening *Rana clamitans* body-length when the tadpoles were immersed in these compounds for ten days. An estimation of relative potency indicated that triiodothyronine was approximately 3.8 times more effective than thyroxine.

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Acknowledgment

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C. J. Shellabarger and J. T. Godwin

Division of Pathology
Medical Department
Brookhaven National Laboratory
Upton, Long Island, New York

Addendum: While this paper was in press similar results were reported by P. C. J. Roth (*Compt. rend. Soc. de Biol.* **147**: 1140, 1953).

THE FAILURE OF LARGE QUANTITIES OF TESTOSTERONE PROPIONATE TO ACTIVATE LOW DRIVE MALE GUINEA PIGS[1]

Numerous data collected during studies of the rat and rabbit are taken in support of the view that strength of sex drive is proportional to supra-maintenance levels of androgen (Stone, 1938; Beach, 1940, 1942a and b, 1944; Beach and Holtz-Tucker, 1949; Cheng and Casida, 1949; Cheng, Ulberg, Christian and Casida, 1950; Kagan and Beach,

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1953; Kinsey, Pomeroy, Martin and Gebhard, 1953). It is clear however even from these reports that supplementary testosterone propionate frequently fails to increase the drive of sexually impotent or sluggish animals. It is to be noted in addition that studies of the bull and guinea pig have been negative. Injections of testosterone propionate into three irregularly breeding bulls gave unsatisfactory results (Hart, Mead and Regan, 1946). The strength of sex drive in male guinea pigs was not raised above precastrational levels when four times the amount sufficient to maintain the precastrational level was given (Grunt and Young, 1952). This was true whether the precastrational drive was low, medium or high. It was concluded that in the male guinea pig strength of sex drive is not proportional to supra-maintenance quantities of testosterone propionate. In order to check the point further, a larger amount of this hormone was administered under conditions which extended the scope of the observations reported earlier.

Twelve males, 11 months of age, were selected. The average score in 10 preliminary tests with estrous females was 4.3 (range 3.4 to 5.6) on a scale from 0.0 to 20.0. Only 3 of the 12 males had achieved ejaculation and these in only 5 of 30 tests. The animals were divided into three groups equated for their performance in the preliminary tests. Each group consisted of 3 non-ejaculating males and 1 sluggish male that ejaculated only infrequently. Animals in group 1 were castrated 3 weeks after the preliminary tests and given daily injections of 500γ testosterone propionate per 100 gm. body weight for 1 month beginning 1 week after castration.[2] This quantity of hormone was selected because it is at least 20 times the amount (12.5 to 25γ) that will restore the precastrational level of sex drive (Grunt and Young, 1952). Animals in group 2 remained intact but received similar injections. Group 3 animals were untreated. All tests during the preliminary and retest periods were concurrent and approximately 3 days apart. They were given under the standard conditions of caging and scoring described elsewhere (Grunt and Young, 1952).

The scores achieved by the males in this study are shown in Figure 1. It will be seen that the experimental animals showed no significant gain in sex drive following the injection of at least 20 times the amount of testosterone propionate necessary to restore precastrational levels; furthermore, non-ejaculating animals did not become ejaculators. On the contrary, there was a trend toward a decrease in strength of sex drive in all groups, a result which is not inconsistent with the earlier observation that by the end of a 41-week period approximately 18% of the original drive had been lost (Grunt and Young, 1952). It seems therefore that within the limits of androgen therapy maintained (nearly 5 mg. per day for each animal), there is no evidence that supra-maintenance quantities of testosterone propionate increase the sex drive of isolated adult male guinea pigs.

Inasmuch as these animals showed no improvement, it is unlikely that their low drive was a consequence of androgenic insufficiency. The explanation for their inferior performance must lie elsewhere and it has been suggested that the character of the behavior is determined in a large part by the nature of the soma or substrate on which the hormones act rather than by the amount of hormone, provided of course the threshold necessary for the somatically determined level has been exceeded (Grunt and Young, 1952).

Acceptance of this hypothesis prompts us to suggest that the effectiveness of supra-maintenance quantities of testosterone propionate without regard for the basis of pre-treatment sexual performance might well be questioned until it has been demonstrated 1) that the animals were not suffering from androgenic insufficiency, and 2) that step-

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[2] Testosterone propionate was supplied by Ciba Pharmaceutical Products, Inc.

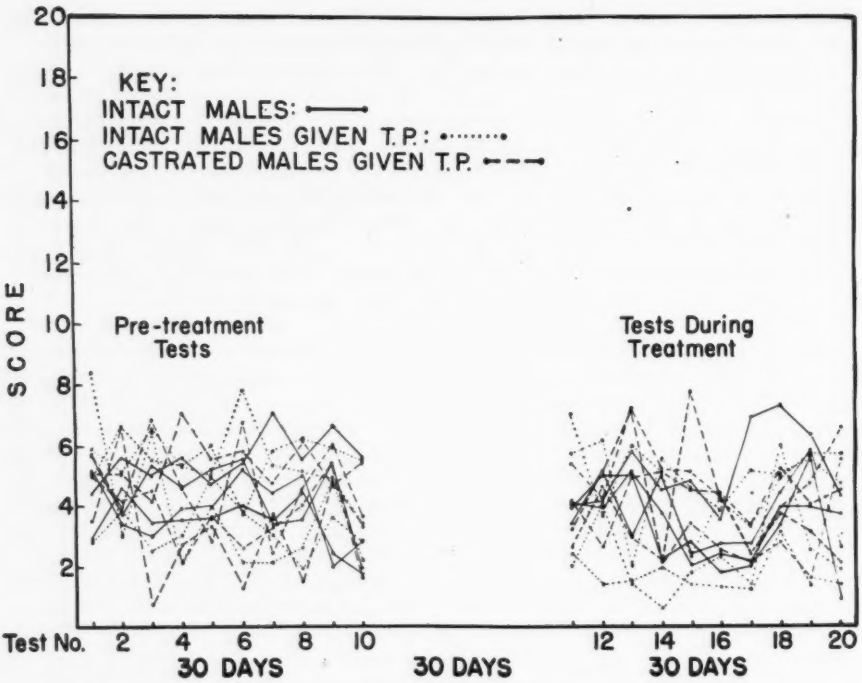

FIG. 1. Strength of sex drive in 8 low drive male guinea pigs before and during treatment with 500γ testosterone propionate per 100 gm. daily (Groups 1 and 2) and in 4 controls (Group 3).

wise increases in supra-maintenance amounts of testosterone propionate result in stepwise increases in drive. To the best of the writers' knowledge, the reports on which such claims are based have not met both of these conditions. As matters stand at the moment, therefore, there are reasons for doubting that large quantities of testosterone propionate *per se* cause intense and mature sexual behavior. It seems more likely that androgen is necessary for the development and maintenance of mature sexual behavior (Moore and Price, 1938; Stone, 1939; Beach and Holz, 1946), but at a level peculiar to the individual animal (Riss and Young, 1954).

SUMMARY

The strength of sex drive was not raised above the pretreatment level when low drive male guinea pigs were injected with more than 20 times the threshold quantity (amount that restores sex drive to the precastrational level) of testosterone propionate.

WALTER RISS and WILLIAM C. YOUNG

Department of Anatomy
University of Kansas
Lawrence, Kansas

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THE 1954 ANNUAL MEETING

The Thirty-sixth Annual Meeting of The Endocrine Society will be held in the Sir Francis Drake Hotel, San Francisco, California, Thursday, Friday, and Saturday, June 17–18–19, 1954.

Dr. Roberto F. Escamilla, 655 Sutter Street, San Francisco, California, is in charge of the local arrangements for the meeting.

All Scientific Sessions will be held in the Sir Francis Drake Hotel. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The annual dinner is scheduled for Friday, June 19, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

Hotel reservations can be made through the San Francisco Convention and Visitors' Bureau, Room #300, Civic Auditorium, San Francisco, California. They should be accompanied by the official AMA application form which appears during December of 1953 in JAMA.

Arrangements have been made for members of this society who do not belong to the AMA to be assigned rooms during the days of the meeting of the Endocrine Society. Applications from non-members of the AMA should specify dates of arrival and departure and be accompanied by a deposit of $10 per room.

Confirmations will not be sent until after February 1, 1954, but applications should be made promptly in order to insure the reservation.

Those wishing to present papers, which will be limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. George W. Thorn, Peter Bent Brigham Hospital, Boston, Massachusetts, not later than February 1, 1954. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes nor acknowledgements to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)."

The principal degree, e.g., M.D., of each author should be given after his name.

Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter-perfect, since there will be no opportunity for proof reading by authors.

1954 AWARD AND FELLOWSHIPS

The award and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts, in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

The Ayerst, McKenna and Harrison Fellowship

The Ayerst, McKenna and Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin and in 1948 to Dr. Ernest M. Brown, Jr. Dr. Brown was re-elected for the Fellowship in 1949. The 1950 Fellowship was awarded to Dr. Lawrence E. Shulman and in 1952 to Dr. John C. Laidlaw. The Fellowship will be in the amount of $5,000 and will be given on alternate years.

The Schering Fellowship in Endocrinology

This Fellowship will not be awarded in 1954 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1955.

AWARD

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishment of an investigator, not more than thirty-five years of age, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman; in 1953 the Award was presented to Drs. Sidney Roberts and Clara Szego. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

Nominations

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 1, 1954.

AMERICAN GOITER ASSOCIATION

The next annual meeting of the American Goiter Association will be held at the Somerset Hotel, Boston, Massachusetts, April 29, 30 and May 1, 1954.